DEFECTIVE RNAs OF SEMLIKI FOREST VIRUS

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Chapter 8 Analysis of DI SFV *in vivo*

SUMMARY

This thesis describes an investigation into the mouse-protecting nucleotide sequences of defective interfering (DI) Semliki Forest virus (SFV) RNA was extracted from tissue culture preparations of DI SFV and reverse transcribed. Putative DI SFV cDNA was amplified by the polymerase chain reaction using primers specific for the termini of the virion RNA. A number of molecular clones were constructed from the products of amplification and the nucleotide sequences of two of these clones were determined (pSFVDI-6: 2146 nts and pSFVDI-19: 1244 nts). Both pSFVDI-6 and pSFVDI-19 were derived from three non-contiguous regions of the SFV genome comprising the 5' and 3' termini and part of the nsP2 coding region. RNA transcribed from these clones was transfected into SFV-infected BHK-21 cells to produce genetically homogeneous DI SFV preparations. These preparations were stable on serial passage and interfered with virus multiplication *in vitro*. The transfection technique was also used in a preliminary investigation of the regulatory elements of the SFV genome. A 388-nucleotide region within the nsP2 gene of SFV was tentatively defined as containing all or part of a packaging signal since DI SFV clones lacking this region were not propagated as virions.

To determine the biological activity of the cloned DI SFV preparations *in vivo* they were mixed with 10 LD_{50} SFV and inoculated into adult mice by the intranasal route. The DI SFV preparation derived from pSFVDI-19 typically conferred 75% protection against the lethal encephalitis that normally follows infection with SFV, whereas the DI SFV preparation derived from pSFVDI-6 was non-protecting. However, it should be noted that the concentration of DI SFV in these cloned preparations was not standardised. Modulation of infection *in vivo* was independent of the antigenic load and mice were susceptible to subsequent lethal challenge. A preliminary experiment suggested that propagation of DI SFV genomes was cell-specific because genomes derived from pSFVDI-19, but not pSFVDI-6, could be detected in mouse brain tissue following intracerebral coinoculation of SFV with the cloned DI SFV preparations.

DECLARATION

All the work described in this thesis was conducted by myself, with the exception of the mouse inoculations which were performed by Professor N. J. Dimmock. All sources of information and materials have been acknowledged either in the text or by means of reference. None of the work described in this thesis has been used in any previous application for a degree.

Michael Thomson

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BHK	baby hamster kidney
CEF	chick embryo fibroblast
CNS	central nervous system
c p e	cytopathic effect
CPV	cytopathic vacuole
DI	defective interfering
FCS	foetal calf serum
LD_{50}	50% lethal dose
m o i	multiplicity of infection
MHV	mouse hepatitis virus
NCS	newborn calf serum
ns	non-structural
-p	passage
PCR	polymerase chain reaction
p f u	plaque-forming units
p i	post-infection
RT-PCR	reverse transcription-PCR
SFV	Semliki Forest virus
$TCID_{50}$	50% tissue culture infectious dose
ts	temperature-sensitive
VSV	vesicular stomatitis virus

CHAPTER 1

GENERAL INTRODUCTION

1. SEMLIKI FOREST VIRUS

(a) Classification

Semliki Forest virus (SFV) was first described by Smithburn and Haddow (1944). Originally it was classified as an arbovirus because it infects vertebrates and is transmitted by a mosquito vector. It has since been classified in the genus alphavirus (formerly known as the group A arboviruses), of the family Togaviridae (Matthews, 1982).

The togavirus family consists of animal viruses which are enveloped and possess a single RNA genome of positive polarity. Togaviruses therefore belong to group IV of the Baltimore classification of viruses (Baltimore, 1971). Besides the alphaviruses, this family includes the genera pestivirus (e.g. hog cholera), arterivirus (e.g. equine arteritis virus) and rubivirus (e.g. rubella virus).

To date, about 25 alphaviruses have been identified, many of which are important veterinary and human pathogens (Chamberlain, 1980). Alphaviruses were originally grouped according to serological data (Calisher and Karabatsos, 1988). More recently, computer analysis of protein sequences has been used to construct phylogenetic trees. Levinson *et al.* (1990) used such an analysis to divide several alphaviruses into three subgroups: the Semliki Forest virus subgroup (SFV, O'nyong-nyong virus and Ross River virus), the eastern encephalitis subgroup (eastern equine encephalitis virus and Venezuelan equine encephalitis virus) and the Sindbis virus subgroup.

(b) Epidemiology

SFV was first isolated from female *Aëdes abnormalis* mosquitoes caught in Bundinyama, western Uganda (Smithburn and Haddow, 1944). Serological studies have shown the virus to be geographically dispersed (Calisher and Karabatsos, 1988), and analysis of phylogenetic trees suggested that alphaviruses originated in the New World (Levinson *et al*, 1990).

SFV has been isolated from mice, birds and mosquitoes, and neutralising antibodies have been found in humans (Chamberlain, 1980). Willems *et al* (1979) reported a case of a laboratory technician developing a lethal encephalitis following infection with SFV and in 1990, Mathiot *et al* identified SFV as responsible for several cases of febrile illness in humans in Central African Republic. However, no other cases of clinical illness in humans following infection with SFV have been reported to date.

(c) Pathogenesis

(i) Progress of infection

SFV was first identified from the ability of mosquito extracts to cause a lethal encephalitis in mice after intracerebral inoculation (Smithburn and Haddow, 1944). The disease was characterised by a roughening of the coat followed by hind limb paralysis, muscular twitchings and convulsions. The original virus isolate was serially passaged in mouse brain. Early passages, from which most laboratory strains are derived, caused death after six days. After about 40 passages, the mean

time of death was 2 days. Hence the virus was highly neurotropic for the central nervous system (CNS). Inoculation by the subcutaneous or intraperitoneal routes delayed the onset of symptoms by one to two days, but there was no difference in the progress of the disease. SFV was shown to be lethal for rabbits, guinea-pigs and rhesus monkeys after intracerebral inoculation (confirmed by Bradish *et al*., 1971).

Seamer *et al* (1967) found that following peripheral inoculation, SFV multiplied in the spleen, liver, lymph nodes and CNS. However, histopathological lesions were only present in the CNS. Pathak and Webb (1974) used electron microscopy to study the mechanism by which SFV enters the CNS. They concluded that the virus was transported across the blood-brain barrier either in coated vesicles or by actively migrating leukocytes.

To date, no receptor for SFV expressed on neural cells has been identified. However, Ubol and Griffin (1991) used an anti-idiotypic antibody to identify a putative Sindbis virus receptor on mouse neural cells. This receptor was a protein that was also detected in murine tissue culture cells but rarely in human cell lines. In addition, the protein was regulated in the development of the nervous system. It was concluded that such a receptor may contribute to the age-dependent susceptibility of mice to fatal encephalitis induced by alphaviruses.

(ii) Virulence

A number of studies have attempted to determine the properties of SFV responsible for virulence. Bradish *et al* (1971) studied the virulence of several

strains of SFV inoculated by the intraperitoneal, respiratory or intracerebral routes in different hosts. Suckling mice were susceptible to all strains, while adult mice were only killed by so-defined virulent strains. The transition to non-susceptibility to avirulent strains occurred at about 16 days of age, after which the virus conferred protection to subsequent challenge (Bradish *et al*., 1972). Puaztai *et al* (1971) showed that after intraperitoneal inoculation of weanling mice with virulent or avirulent strains of SFV (V13 and A7), a viraemia was induced followed by brain invasion. The initial stages of the disease were identical for both virulent and avirulent strains (confirmed by Bradish and Allner, 1972). However, V13 was detected in the brain 24 hours before A7 and titres increased to death, while titres of A7 reached a peak and then declined.

Histopathological lesions have been observed in the CNS following infection of adult mice with both virulent and avirulent strains of SFV. However, the cellular effects are different for the two strains. Electron microscopic studies have shown that following infection of adult mice with the A7(74) strain there is no virus maturation and viral nucleocapsid material accumulates in neurons. The infection results in a subacute demyelinating meningoencephalomyelitis with little neuronal destruction or infection of oligodendrocytes and is followed by remyelination and complete recovery (Pathak *et al*., 1976; Pathak and Webb, 1983, 1988; Khalili-Shirazi *et al*., 1988; Fazakerley and Buchmeier, 1992). In contrast, lethality of virulent strains is related to necrosis of neurons and oligodendrocytes (Atkins and Sheahan, 1982; Atkins *et al*., 1985, 1990; Smyth *et al*., 1990). In addition, the avirulent A7 strain has been shown to induce abortion following peripheral infection of pregnant mice (Atkins *et al*., 1982). Teratogenesis was shown

subsequently to result from virus infection of cells originating in the mesenchyma (Mabruk *et al*., 1988, 1989)

One feature of infection by several neurotropic viruses, including Russian spring-summer encephalitis, St Louis encephalitis and Dengue-2 viruses, is the induction of smooth membrane proliferation in the CNS (Murphy, 1980). Electron microscopic studies have shown that acute encephalitis of adult mice resulting from infection with the virulent L10 strain of SFV is also associated with the development of numerous membrane vesicles, from which mature virus particles bud. In contrast, infection of adult mice with the A7(74) strain does not result in significant membrane proliferation and mature virus particles have not been detected in the brain (Pathak *et al*., 1976, Pathak and Webb, 1978). This association between virulence and membrane proliferation has been confirmed by studies in which adult mice were treated with gold sodium thiomalate (GSTM) before infection with avirulent SFV. Virus titres in the brain were shown to be significantly increased and the infection was lethal (Bradish *et al*., 1975, Mehta and Webb, 1987). Subsequent studies showed that GSTM induces membrane proliferation and depresses peritoneal macrophage function (Mehta and Webb, 1990, Mehta *et al*., 1990). In addition, it has been suggested that the lethality of avirulent SFV for neonatal mice is associated with membrane proliferation. In contrast to adult mice, the brain cells of neonatal mice are still developing and there may be sufficient membrane proliferation for virus maturation (Mehta *et al*., 1990).

(iii) CNS demyelination

Avirulent SFV has been used as a model for investigating demyelinating virus diseases of humans, and in particular, multiple sclerosis (Atkins *et al*., 1990, reviewed in Fazakerley and Buchmeier, 1993). A number of studies have suggested that demyelination by avirulent SFV is immune-mediated. Berger (1980) showed that following infection with the A7(74) strain, demyelination was more severe in immunocompetent mice than in nude mice. This supported previous work that suggested demyelination was T-cell dependent (Jagelman *et al*., 1978). In addition, Jenkins *et al*. (1988) showed that infection of T-cell deficient nude mice with the avirulent A7(74) strain did not result in optic nerve demyelination. Fazakerley and Webb (1987a) showed that adoptive transfer of syngeneic spleen cells to A7(74)-infected athymic mice restored the lesions of demyelination in the CNS. However, this did not occur following peripheral transfer of anti-SFV immune serum and demyelination could be prevented by total body irradiation (Fazakerley and Webb, 1987b). Fazakerley and Buchmeier (1993) suggested that demyelination is mediated through a T-cell response to viral determinants on the cell surface or to an autoantigen.

(iv) The molecular basis of virulence

In an attempt to determine the molecular basis of virulence, Barrett *et al*. (1980) isolated and characterised a number of mutants of SFV (L10 virulent strain) showing altered virulence in weanling mice. Loss of neurovirulence was shown subsequently to be associated with a loss of the ability either to enter the brain or to damage neurons (Atkins and Sheahan, 1982). Two mutants (M9 and M136) had

similar host range and molecular properties to the avirulent A7 strain (Atkins, 1983). Subsequently, Hearne *et al.* (1987) isolated four temperature-sensitive mutants of the A7 strain that had altered pathogenicity for mouse embryos. These mutants had defects in RNA synthesis that were more extreme than that expressed by the A7 strain (Woodward and Smith, 1979).

Recently, investigation of the molecular basis of alphavirus pathogenesis has been facilitated by the construction of infectious clones (SFV: Liljeström *et al.*, 1991; Sindbis: Rice *et al.*, 1987). Glasgow *et al.* (1991) showed that virus derived from the SFV-derived clone had similar pathogenic properties to the virulent L10 strain. Two mutations in the p62 envelope protein region (p62 is a precursor of the E2 and E3 glycoproteins; see section 1d) reduced the rate of multiplication of the virus in mouse brain and extended the mean time of death following intranasal inoculation of 40-day-old mice. Similar work with Sindbis virus has shown that defined mutations affecting pathogenesis occur within the genes encoding the membrane glycoproteins E1 and E2 (Lustig *et al.*, 1988; Polo *et al.*, 1988; Polo and Johnston, 1990; Tucker and Griffin, 1991).

(d) Structure

Figure 1.1 shows a schematic diagram of SFV. SFV is an enveloped virus with an icosahedral nucleocapsid (Kääriäinen and Söderlund, 1978). The nucleocapsid contains a single molecule of positive-stranded RNA of sedimentation coefficient 42S (Friedman *et al.*, 1966). Electron microscopic studies have shown that the nucleocapsid is surrounded by a lipid bilayer with an average radius of 23 nm. The lipid membrane contains 80 spike proteins extending to an outer radius of about

SEMLIKI FOREST VIRUS

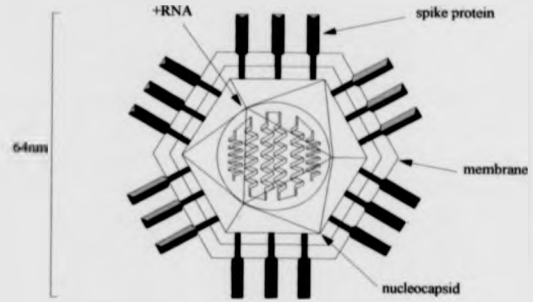


Figure 1.1 Schematic diagram of a Semliki Forest virus particle

The diagram of SFV is reproduced from Branden and Tooze (1991). The particle is about 64 nm in diameter. A single 42S RNA molecule is contained within a T=4 icosahedral nucleocapsid that comprises a single capsid protein. The nucleocapsid is surrounded by a lipid bilayer of host-cell origin. 240 spike protein structures are arranged as trimers in the lipid membrane and are composed of the E1, E2 and E3 structural proteins.

32 nm. The spikes are organised as a lattice with a triangulation number T = 4 (Vogel *et al*., 1986, Fuller, 1987).

The nucleocapsid core consists of capsid protein with a molecular weight (M_r) of 30,000 (Söderlund *et al*., 1979). The capsid protein contains 267 amino acids and has clusters of basic amino acids and prolines in the N-terminal third of the polypeptide chain which are suspected to be involved in binding to the 42S RNA (Garoff *et al*., 1980a, b). Biochemical studies have shown that each spike protein consists of two glycoproteins that span the lipid bilayer (E1, M_r=49,000 and E2, M_r=51,000) and a third (E3, M_r=10,000) that is associated with the external domains of E1 and E2. At present it is not known how E3 is bound to the spike protein. The viral membrane can be solubilised with nonionic detergents into E2E1 heterodimeric structures. This and other studies have suggested that the SFV spike is organised as a trimer of the E2E1 heterodimer (Simons *et al*., 1973, Ziemiecki and Garoff, 1978, Wahlberg *et al*., 1989, Anthony and Brown, 1991, Levy-Mintz and Kielian, 1991, Wahlberg *et al*., 1992).

The E1 and E2 glycoproteins are organised in the lipid bilayer with the carboxy (C) -terminus on the inside. The haemagglutinating activity of the virus is a function of the E1 subunit (Chanas *et al*., 1982) and E2 has the determinant for neutralisation (Boere *et al*., 1983). Two arginine residues at the C-terminus of E1 and 31 amino acid residues at the C-terminus of E2 are located on the internal side of the membrane. The interaction of the spike protein with the nucleocapsid is therefore thought to occur through the C-terminus of E2 (Garoff *et al*., 1980a). Recently, Barth *et al* (1992) showed that the C-terminal peptide of SFV had no major role to play in the multiplication of the virus in cells of vertebrate origin.

A number of studies have provided biochemical evidence for spike protein-nucleocapsid interactions of SFV and Sindbis virus (SFV: Garoff and Simons, 1974; Helenius and Kartenbeck, 1980; Sindbis: reviewed in Schlesinger and Schlesinger, 1986). The work of Vaux *et al.* (1988) and Kail *et al.* (1991) showed by means of anti-idiotypic antibodies that the C-terminal domain of E2 has structural complementarity to the nucleocapsid and that the C-terminal 8 amino acids of E2 were a signal for nucleocapsid interaction. However, Suomalainen and Garoff (1992) have since demonstrated that the anti-E2 antibody used in this work was not specific for nucleocapsid protein as claimed, but recognised a component of the viral replication machinery. More reliable evidence for spike protein-nucleocapsid interaction was provided by Metsikkö and Garoff (1990), who showed that synthetic peptides comprising the C-terminal domain of E2 bound efficiently to nucleocapsids *in vitro*. Furthermore, oligomers of these peptides interacted in preference to monomers, suggesting that the interaction with the viral nucleocapsid occurs through complexes of the E2 protein.

(e) Genome structure

(i) Primary structure

Figure 1.2a shows a diagram of the protein-coding regions of the SFV genome. The 42S RNA is 11,442 nucleotides in length (M_r= 3.95 x 10^6) and has a base composition of 26.7% A, 20.1% U, 27% G and 26.2% C (Garoff *et al.*, 1980a, 1980b; Riedel *et al.*, 1982; Takkinen, 1986). At the 5' terminus is a 7-methyl guanosine cap structure, which has the same nucleotide sequence as that for the

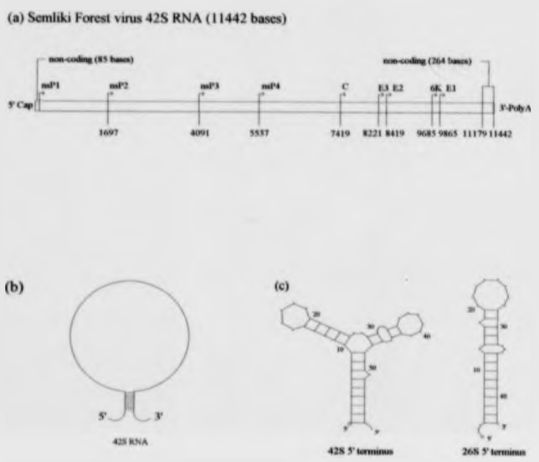


Figure 1.2 Primary and secondary structures of SFV RNA

(a) shows the protein coding regions of the 42S RNA **(b)** shows a schematic diagram of the panhandle structure of the 42S RNA observed by electron microscopy **(c)** is reproduced from Rietben-Bloemheuvel (1992) and shows the proposed secondary structure model for the 5' untranslated regions of the 42S and 26S RNAs of SFV

Sindbis virus genome (SFV: Pettersson *et al*., 1980, Sindbis: Hefti *et al*., 1976, Dubin *et al*., 1977). At the 3' terminus of SFV RNA and other alphavirus genomes is a poly(A) tract of approximately 80 nucleotides (Armstrong *et al*., 1972, Eaton and Faulkner, 1972, Clegg and Kennedy, 1974). The 50 nucleotides at the 3' terminus of SFV RNA have an 80% A + U content. It has been suggested that this region may serve as a polyadenylation signal (Ou *et al*., 1982b).

The 85 nucleotides at the 5' terminus and the 264 nucleotides at the 3' terminus of the SFV genome are not translated (Garoff *et al*., 1980b, Takkinen, 1986). There is an open reading frame coding for a 2431 amino acid polyprotein which extends from an AUG codon at nucleotides 86-88 to a UAA termination codon at nucleotides 7379-7381. Non-structural proteins (nsP1-4) are translated from this region (see section 1g-*v*). Structural proteins are translated from 26S RNA, which is derived from the 3' 4074 nucleotides of the 42S RNA in the infected cell (see section 1g-*vi*, Takkinen, 1986). There is a 13-nucleotide overlap of the 26S RNA with the 42S genome and 51 nucleotides at the 5' terminus of the 26S RNA are not translated. The structural proteins are translated in a different reading frame to that used for translation of the non-structural proteins (Riedel *et al*., 1982, Takkinen, 1986).

(ii) Secondary structure

Electron microscopic studies of Sindbis virus and SFV RNAs showed that the molecules can circularise and form panhandle structures (Figure 1.2b, Hsu *et al*., 1974, Kennedy, 1976, Frey *et al*., 1979). Ou *et al*. (1983) suggested that the base-pairing of the panhandle occurred between complementary regions of 10

nucleotides located 190 nucleotides within the 5' terminus and 241 nucleotides within the 3' terminus. The extreme termini of the RNA were not suspected to be involved in base-pairing themselves.

A number of regions of alphavirus RNAs have been implicated as important to the regulation of replication. Ou *et al.* (1983) suggested that stem-loop structures can be formed from about the first 40 nucleotides of several alphavirus genomes and they showed that a 51 nucleotide region was conserved at the 5' terminus of the 26S RNA. These regions were suspected to have a role in the regulation of RNA transcription. In addition, Ou *et al.* (1982a) suggested that a 21 nucleotide region at the junction between the 26S RNA and the non-structural coding sequences was required for the initiation of transcription of the 26S RNA. Although these conclusions were not based on experimental evidence, Levis *et al.* (1990) showed that this region could direct the transcription in the infected cell of subgenomic RNA from a defective interfering Sindbis virus genome. Konings *et al.* (1987) analysed leader sequences from 50 eukaryotic mRNAs, including SFV RNA, and showed that the majority had a common conformation at the 5' region. Experimental data have suggested that such regions are important for translation from the correct start codon and for regulation of viral protein synthesis in the infected cell (van Duijn *et al.*, 1988; Berben-Bloemheuvel *et al.*, 1992). Figure 1.2c shows the computer-predicted secondary structure model proposed by Berben-Bloemheuvel *et al.* (1992) for the 5' termini of the 42S and 26S RNAs. In this model the cap structure of the 42S RNA is masked and may impede translation in the infected cell, whereas the 26S RNA cap is free to interact with cap-binding protein. This may explain why the 26S RNA is able to escape the shut-off of host protein synthesis (see section 1g-*iii*).

(f) Growth *in vitro*

SFV grows in a wide variety of cell types, including several mammalian, human and mosquito lines, and is readily assayed by plaque formation (Henderson and Taylor, 1960). In addition, it grows over a wide temperature range of 20-40°C (Burge and Pfefferkorn, 1966). For these reasons, and because it is relatively non-pathogenic for humans, SFV has been used extensively as a model for studying the multiplication of enveloped RNA viruses.

Early studies on the growth of SFV in chick embryo fibroblasts (CEF) showed that the virus replicated entirely in the cytoplasm (Taylor, 1965; Acheson and Tamm, 1967). At about 3 hours after infection of CEFs at 37°C, the rate of production of SFV in the culture medium increased exponentially to a maximum of 200 plaque-forming units/cell/hour at 5-9 hours after infection. Cytopathic changes were observed 6 hours after infection and cells were rounded and detaching from the surface of the growth vessel by 12 hours. The maximum cytopathic effect coincided with the cessation of virus production. Electron microscopic studies showed the appearance of unique membranous structures, or cytopathic vacuoles (CPV), following infection of CEFs and other cell lines with SFV (Acheson and Tamm, 1967; Erlandson *et al*., 1967; Friedman and Berezesky, 1967; Grimley *et al*., 1968; Friedman *et al*., 1972). Two morphologically distinct CPVs were identified. The first was produced 1-2 hours after infection (CPV-I) and the second (CPV-II) was produced late in the infectious cycle. Grimley *et al* (1968) showed that CPV-Is were loci of RNA synthesis and more recently, Froshauer *et al* (1988) and Peränen and Kääriäinen (1991) have shown that they are endosomes and lysosomes associated with the replicative proteins nsP3 and nsP4.

While the growth of SFV and other alphaviruses in vertebrate cell lines results in cell lysis, infection of mosquito cell lines is often persistent with little or no cytopathic effect (Davey *et al*., 1973, Igarishi *et al*., 1977, Tooker and Kennedy 1981). The nature of the host cell components that influence the growth of alphaviruses in vertebrate and invertebrate cells has yet to be elucidated, but Baric *et al* (1983) suggested that the growth of Sindbis virus in these cell lines required host cell transcription and presumably protein synthesis.

(g) Infectious cycle

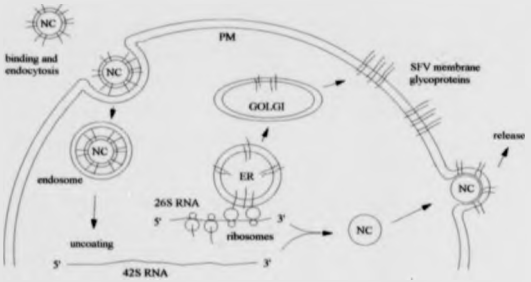
(i) Overview

Figure 1.3 shows a schematic diagram of the infectious cycle of SFV. Each stage of the cycle is described in detail below.

(ii) Binding and endocytosis

SFV binds to a receptor on the surface of cells through its spike proteins and is internalised by endocytosis in coated vesicles (Helenius *et al*., 1980, Marsh *et al*., 1983, Haas *et al*., 1989).

At present it is not known which component of the host cell membrane is required for the attachment of alphavirus particles. Alphaviruses can infect a wide range of cell types, both vertebrate and invertebrate, and must therefore recognise either different receptors or a receptor that is ubiquitous. Helenius *et al* (1978) showed



Key: NC = nucleocapsid, PM = plasma membrane, ER = endoplasmic reticulum

Figure 1.3 The infectious cycle of SFV (adapted from Garoff *et al*., 1982)

The virus binds to the host cell receptors through its spike proteins and enters the cell by endocytosis. The virus is transported to endosomes in coated pits where the acidic pH results in a conformational change in the spike proteins. This induces fusion between the viral and endosomal membranes and the release of the nucleocapsid into the cytoplasm where it is uncoated. The 42S RNA acts as messenger for the translation of viral replicase proteins which transcribe new genomes and subgenomic 26S RNA. The 26S RNA encodes the structural proteins of the virus. The nucleocapsid assembles in the cytoplasm and the spike proteins become inserted in the membrane of the rough endoplasmic reticulum and are transported through the Golgi apparatus to the plasma membrane of the cell. Finally the spike proteins are incorporated into the viral envelope and the mature virion is released by budding at the cell surface.

that SFV could bind to antigens of the major histocompatibility complex of human and murine cells. However, studies by Oldstone *et al* (1980) showed that infection by SFV could still occur in the absence of these antigens. Baby hamster kidney (BHK)-21 cells have been shown to bind approximately 5×10^4 SFV particles/cell at 4°C under saturating conditions (Fries and Helenius, 1979). Helenius *et al* (1980) showed that SFV binds preferentially to microvilli on BHK cells but the nature of the receptor was not determined. Recently, Wang *et al* (1992) obtained a monoclonal antibody that blocked the attachment of Sindbis virus to BHK-21 cells and partly blocked attachment to mosquito cells. The antibody recognised a cell surface protein that bound to basement membrane laminin and was highly conserved among mammals. They suggested that the ubiquity of this receptor was responsible for the wide host range of Sindbis virus.

(iii) Membrane fusion and entry into the cytoplasm

Following internalisation, SFV is transported in coated vesicles to the endosomes where the acidic pH induces a conformational change in the spike proteins. This results in fusion between the viral and endosomal membranes and precedes entry of the nucleocapsid into the cytoplasm (White and Helenius, 1980, Helenius *et al*, 1982, Kielian and Helenius, 1985, Wahlberg and Garoff, 1992).

Fusion of SFV particles to the host membrane is mediated through the E1 subunit of the spike proteins (Omar and Koblet, 1988, Levy-Mintz and Kielian, 1991). This fusion reaction is known to require the presence of cholesterol in the target membrane (Kielian and Helenius, 1984, Phalen and Kielian, 1991). Wahlberg and Garoff (1992) showed that the E2E1 heterodimeric association of the spike protein

(see section 1d) is destabilised soon after the virus enters the endocytic pathway. Exposure of the spike protein to mildly acidic pH causes the E1 subunit to dissociate from the E2 protein and form a homotrimeric complex (Wahlberg *et al*, 1992). Virus penetration and fusion processes are inhibited by a monoclonal antibody directed specifically to this trimeric form of E1, which is suspected therefore to represent the SFV fusion protein (Wahlberg and Garoff, 1992, Wahlberg *et al*, 1992).

Electron microscopic studies have shown that nucleocapsids appear on the cytoplasmic side of the endosomal membrane immediately after fusion. These rapidly disappear from view, suggesting that they are disassembled as soon as they enter the cytoplasm (Helenius, 1984). Host cell protein synthesis is shut off about 4 hours after infection with SFV. The capsid protein is suspected to be important in this process, but the precise mechanisms involved have not been determined (van Steeg *et al*, 1984, Elgizoli *et al*, 1989).

(iv) Uncoating

The passage of the nucleocapsid through acidic endosomes is not thought to be important to the uncoating process (Stubbs *et al*, 1991). Uncoating of Sindbis virus has been shown to occur through association with ribosomes (Wengler and Wengler, 1984, Wengler *et al*, 1984). Recently, Singh and Helenius (1992) showed that a similar association occurs with the SFV nucleocapsid. Ribosomes were shown to bind to capsid proteins and release them from the nucleocapsid in an irreversible, non-catalytic process. The precise interaction of the capsid protein with ribosomal RNA is not known at present, but Choi *et al* (1991) have

suggested that the interaction may be mediated through the amino terminal segment of the capsid protein, which is exposed on the surface of the nucleocapsid.

(v) Synthesis and function of the non-structural proteins

Following uncoating, the 42S RNA is released into the cytoplasm where it acts as messenger for the translation of non-structural (ns) proteins. The 5' two-thirds of the genome is translated as a polyprotein (nsP1234, see figure 1.2a) which is cleaved proteolytically to yield four mature proteins (nsP1-nsP4; M_r = 60,000, 89,000, 52,000, 69,000 respectively). All four proteins are required for replication of the 42S RNA and are thought to become associated as a replication complex in the infected cell (Takkinen, 1986; Kaariainen *et al*., 1987; Barton *et al*., 1991; Lemm and Rice, 1993). A number of studies have revealed short-lived intermediate polypeptides in infected cells. These studies suggested that the nsP1234 polyprotein is processed through two different pathways to yield either nsP123 + nsP4 or nsP12 + nsP34 precursors (Lachmi and Kaariainen, 1977; Lehtovaara *et al*., 1980; Takkinen *et al*., 1990, 1991).

A number of recent studies on Sindbis virus and SFV have suggested functions for the four non-structural proteins produced in the infected cell. nsP1 has been implicated in the synthesis of negative-strand RNA (see section 1g-vi). In addition it has been suggested to be involved in the methylation and capping of positive-strand RNA (Mi *et al*., 1989; Wang *et al*., 1991). nsP2 has two enzymatic activities. The N-terminal half is thought to function as a helicase and the C-terminal half as an autoprotease (Ding and Schlesinger, 1989; Hardy and Strauss, 1989). In addition, nsP2 possesses information that directs itself to the nucleus of

the infected cell. The function of nsP2 in the nucleus is not known, but it has been suggested that it may serve to inhibit host cell DNA and RNA synthesis (Peränen *et al*., 1990).

nsP3 is a phosphoprotein with phosphorylated serine and threonine residues and is associated with the cytopathic vesicles described in section 1f (Frosshauer *et al*., 1988, Peränen *et al*., 1988, Peränen, 1991, Peränen and Kääriäinen, 1991). However, the function of nsP3 has not been determined, although Hahn *et al* (1989) demonstrated that it was essential for RNA synthesis. Finally, nsP4 is thought to function in the initiation and elongation processes of RNA synthesis and possesses autoprotease activity (Sawicki *et al*., 1990, Takkinen *et al*., 1990). For SFV and Sindbis virus, nsP4 is produced in lower amounts in the infected cell than nsP1, 2 or 3 (Keränen and Ruohonen, 1983; Li and Rice, 1989).

(vi) Synthesis of 42S and 26S RNAs

The nonstructural proteins translated from the 42S RNA serve as an RNA-dependent RNA polymerase for the synthesis of new genomes and the subgenomic 26S RNA. The 42S RNA is a template for the production of minus-strand 42S, which in turn is a template for the synthesis of new plus-strand 42S RNA. The 26S RNA is transcribed from an internal initiation site on the negative-strand 42S RNA (Kääriäinen and Söderlund, 1978, Pettersson *et al*., 1980, Sawicki and Sawicki, 1980, Sawicki *et al*., 1981, Riedel *et al*., 1982). The rate of synthesis of plus and minus-strand RNAs increases during the first 3 hours post-infection, after which the synthesis of minus-strand RNA ceases. The synthesis of 42S and 26S RNAs

continues at a constant rate throughout the infectious cycle (Bruton and Kennedy, 1975, Sawicki and Sawicki, 1980)

A number of temperature-sensitive mutants of Sindbis virus have been obtained which fail to shut off the synthesis of minus strand RNA (Sawicki *et al*., 1981, Sawicki and Sawicki, 1986) Sawicki *et al* (1990) showed that a single base change in the nsP4 gene was responsible for the phenotype of three such mutants and suggested that a domain of nsP4 functions to ensure that the minus strand is the preferred template of the replication complex

(vii) Synthesis of structural proteins

All the structural proteins of SFV are synthesised from the 26S RNA as a polyprotein precursor from a single initiation site The polyprotein is synthesised in the order C-p62-6K-E1 (M_r = 33,000, 62,000, 6,000, 50,000 respectively) and individual proteins are released from the precursor by post-translational cleavage (Clegg and Kennedy, 1975, Garoff *et al*., 1980b) The 26S RNA is suspected to be translated more efficiently than the 42S RNA This may be a result of differences in the untranslated regions of these RNAs (Berben-Bloemheuvel *et al*., 1992)

The capsid protein is synthesised in the cytoplasm and is released from the nascent polypeptide by autoproteolysis (Melançon and Garoff, 1987) This event reveals the N-terminal sequence of the p62 polypeptide which targets the p62-6K-E1 polypeptide to the endoplasmic reticulum (ER) All but the C-terminus and membrane anchor sequences are translocated across the ER membrane (Garoff *et al*., 1978) Recent studies have shown that the signal sequence of p62 consists of a

16-amino acid peptide at the N-terminus of the polypeptide (Garoff *et al*., 1990). The p62 signal sequence is glycosylated in the lumen of the ER before the entire p62 sequence has been synthesised, suggesting it is not important for the completion of chain translocation (Garoff *et al*., 1990).

The C-terminus of p62, which remains on the cytoplasmic side of the ER, contains a second signal sequence that is involved in the translocation of the 6K polypeptide and is cleaved by a signal peptidase. Translocation of 6K continues until the anchor sequence becomes inserted into the ER membrane (Liljeström and Garoff, 1991a). The translocation of the E1 polypeptide proceeds in a similar manner, with the C-terminus of 6K serving as a signal sequence which is cleaved by a signal peptidase (Melançon and Garoff, 1986, Liljeström and Garoff, 1991a).

(viii) Fate of the structural proteins

Within the ER the p62 precursor becomes associated as a heterodimer with E1 through its E3 domain (Wahlberg *et al*., 1989, Lobigs *et al*., 1990). This heterodimeric complex is transported via the Golgi network to the plasma membrane of the infected cell (Green *et al*., 1981, de Curtis and Simons, 1988). The 6K protein also associates with the p62E1 complex in the ER and is transported to the plasma membrane. However, the precise role of 6K in virion maturation is unclear. Deletion of 6K from the SFV genome does not prevent transport of the p62E1 complex to the plasma membrane but does result in a significant reduction of virus release (Liljeström *et al*., 1991, Lusa *et al*., 1991).

Before the p62E1 heterodimer reaches the plasma membrane, the p62 component is proteolytically cleaved to yield the E2 and E3 proteins that are present in the mature virion (de Curtis and Simons, 1988, Wahlberg *et al*., 1989). In mosquito cells, this cleavage has been shown to occur before entry into the ER. However, the significance of this is not known at present (Naim and Koblet, 1990). Salminen *et al* (1992) showed that a mutation in the p62 cleavage site prevented maturation of the protein. This did not affect virus release but virions were non-infectious. They concluded that p62 cleavage is required for virus uptake into cells and for promoting membrane fusion in the endosomes. The E1 moiety is also modified after transport through the Golgi network to a pH-sensitive form (Kielian *et al*., 1990). This mature form of E1 permits membrane fusion following entry of the virus into acidic lysosomes (see section 1g-*ii*).

(ix) Assembly and release

Assembly of virion particles requires the association of genomic RNA with capsid protein and subsequent interaction of the nucleocapsid with the spike glycoproteins embedded in the plasma membrane of the infected cell. The 42S RNA but not the 26S RNA is encapsidated, suggesting that a specific interaction occurs between the 5' two-thirds of the genome and the capsid protein (Ulmanen *et al*., 1976). However, the precise nature of this interaction has not been determined. Studies with Sindbis virus showed that the capsid protein can interact with different RNAs to form stable nucleocapsid structures, suggesting that a specific packaging signal is not necessarily required for encapsidation in the infected cell (Wengler *et al*., 1982, Wengler, 1987). However, Weiss *et al* (1989) identified a 500-nucleotide domain within the nsP1 gene that bound efficiently to capsid protein *in vitro*. In

addition, deletion of this domain from a defective interfering genome prevented its encapsidation in the infected cell, suggesting that a packaging signal is needed for nucleocapsid formation. To date, no such signal has been identified for SFV.

SFV and other alphaviruses mature by budding at the cell surface (Acheson and Tamm, 1967; Erlandson *et al.*, 1967). In this process, the nucleocapsid is surrounded by a lipid bilayer envelope containing the viral spike proteins. Recent evidence suggests that budding is driven by a specific interaction of the membrane-bound spike proteins with the nucleocapsid (see section 1d; Suomalainen *et al.*, 1992). Zhao and Garoff (1992) showed that membrane-bound spike proteins degrade rapidly in the absence of nucleocapsid, suggesting an efficient mechanism exists to capture nucleocapsids into budding complexes. However, the nature of this mechanism has yet to be determined.

2. DEFECTIVE INTERFERING VIRUSES

(a) Overview

Defective interfering (DI) viruses have been described for all viruses studied. It is beyond the scope of this thesis to describe them all and the remainder of this introduction will focus mainly on the DI particles of alphaviruses, with reference to other viruses where appropriate. A number of reviews are available on aspects of DI viruses that are not considered here (Lazzarini *et al*., 1981, Perrault, 1981, Holland, 1985, 1990, Barrett and Dimmock, 1986, Nayak *et al*., 1989, Dimmock, 1991, Roux *et al*., 1991).

(i) Definition

Von Magnus (1954) first described the generation of non-infectious or 'incomplete' virus particles by serial passage of high multiplicities of influenza virus in fertilised hens eggs. Following these first observations, evidence accumulated in a number of animal and tissue culture systems for a distinct type of non-infectious virus particle that could interfere with the intracellular replication of homologous virus. Based on this evidence, Huang and Baltimore (1970) defined 'defective interfering' or 'DI' particles as responsible for the interference phenomenon. They also described the non-defective homologous virions as 'standard virus', these are the terms that will be used predominantly in this thesis.

It should be noted that the term 'defective' can apply to any virus that lacks some function essential to autonomous replication and a defective virus does not

necessarily have the capacity to interfere with standard virus propagation. In addition, 'interference' with standard virus propagation is not a property exclusive to DI viruses. For example, satellite RNAs of plant viruses are capable of modulating disease but share little sequence homology with the standard virus genome and thus differ from DI genomes (Francki, 1985).

The distinguishing properties of DI particles are summarised below (adapted from Barrett and Dimmock, 1986) -

- Incapable of autonomous replication, i.e. are defective
- Reduce the yield of standard virus following coinfection, i.e. are interfering *
- Possess a deleted form of the standard virus genome
- Require a functional nucleic acid for interference
- Are antigenically identical to standard virus
- Are enhanced after coinfection with standard virus

* This depends on the ratio of standard to DI virus used in coinfection: with excess standard virus there is no reduction in standard virus titres.

(ii) Significance of DI particles

Most animal viruses have been shown to have associated DI viruses. In addition, DI viruses have been described for plant viruses (Li *et al*., 1989). The ubiquity of DI viruses suggests they have a role in natural infection and confer some evolutionary advantage to the standard virus. Huang and Baltimore (1970) first suggested that DI virus may be important to persistence of viral diseases but the

difficulty in isolating DI virus from naturally occurring infections has impeded the resolution of this hypothesis.

Whatever the natural role of DI viruses, their ability to interfere with standard virus propagation suggests a use as antiviral agents. This concept is supported by the fact that DI viruses are ubiquitous, non-infectious and possess the same structural proteins and therefore have the same cellular tropisms and immunology as standard virus. The potential of DI viruses as antiviral agents has not yet been fully realised, but as will be described below, a number of investigations have shown that DI viruses can modulate the progress of viral disease in experimental infections.

DI viruses have also been used extensively to study the molecular biology of viruses. DI virus genomes are generally a small fraction of the standard genome size and their regulatory elements reside in a relatively short length of nucleic acid. This has facilitated the identification of viral regulatory elements in a number of systems, including Sindbis virus and mouse hepatitis virus (see section 2c).

(b) DI particle structure

(i) Structure

Most DI genomes are unable to encode structural proteins and are therefore dependent on the standard genome for the provision of these. DI particles are therefore considered as containing the same structural proteins as standard virus and being antigenically indistinguishable from standard virus. It is conceivable for a

DI genome to encode truncated structural proteins that are incorporated into the nucleocapsid, but there is no evidence for this

The buoyant density of DI alphavirus particles on a caesium chloride gradient is generally greater than standard virions (Shenk and Stollar, 1973; Bruton and Kennedy, 1976). It was suspected that this was a consequence of virions packaging multiple copies of DI genomes or the DI SFV particles packaged single copies but had more compact nucleocapsids. The latter conclusion is supported by evidence that DI SFV has single-hit u.v.-inactivation kinetics (Barrett *et al*., 1981)

Electron microscopic studies have shown that particles of DI alphavirus are on average smaller than those of standard virus. Following serial passage of Sindbis virus in BHK cells, Johnston *et al* (1975) described the appearance of particles that were 37 nm in diameter compared with 50 nm for standard virions. Although the appearance of these smaller particles correlated with a decrease in virus titre, the larger particles were also suspected to have interfering activity. Later studies by Barrett *et al* (1984b) showed that DI particles of SFV had an average diameter of 47 nm compared with 56 nm for standard virus particles.

The only DI particles that differ obviously in size from standard particles are those of the rhabdovirus, vesicular stomatitis virus (VSV). Electron microscopic studies showed DI VSV as shorter rods than standard virus particles (Hackett, 1964) and later studies showed that the DI particle size was determined by the size of the packaged genome (Holland *et al*., 1976)

(ii) Separation of DI particles from infectious virus

Because of the similarity in size of the particles of DI alphavirus and standard virus, separation of the two has proved difficult. Bruton and Kennedy (1976) could separate DI SFV from standard virus on a caesium chloride gradient, but biological activity was destroyed. Attempts to separate DI SFV from standard virus whilst retaining biological activity using metrizamide or sucrose density gradient centrifugation were unsuccessful (Kääriäinen *et al*., 1981, Barrett *et al*., 1984a). Fortunately the u v -target size of DI SFV genomes is smaller than that of standard virus enabling infectivity to be removed from DI virus preparations without destroying the biological activity of the DI genome (Dimmock and Kennedy, 1978). The DI particles of VSV, being smaller than those of standard virus, can be separated by sucrose density gradient centrifugation (Huang *et al*., 1966).

Recently a novel system has been developed to enable DI VSV to be propagated in the absence of helper standard virus. cDNA clones of each of the five VSV genes and a DI VSV genome were transfected simultaneously into BHK-21 cells harbouring a vaccinia recombinant that expressed T7 RNA polymerase cytoplasmically. Transcription of these clones was mediated within the cells through T7 promoters incorporated at the 5' termini. The 3' termini of the transcripts were generated through autolytic cleavage by a ribozyme that was incorporated downstream of the viral cDNA. The DI RNA transcripts that were generated were replicated and encapsidated by proteins expressed from the cloned virus genes and mature DI particles were obtained in the culture medium (Pattnaik and Wertz, 1990, 1991, Pattnaik *et al*., 1992). With the genomes of SFV now cloned, it may be possible to develop a similar system for the production of helper-

free DI SFV preparations and avoid the need for u.v.-irradiation to remove infectivity.

(c) DI virus genomes

(i) Semliki Forest virus

In early studies, DI SFV generated after nine serial undiluted passages of SFV in BHK-21 cells was shown to contain two positive-strand RNA species that were approximately 20% (about 2 kb) of the standard RNA size (Bruton and Kennedy, 1976). Further work by Bruton *et al.* (1976) showed that these RNAs shared extensive sequence homology and were not derived from the nucleotide sequences of the 26S RNA of SFV. In addition, both RNAs were capped and polyadenylated but were not able to serve as mRNAs. Subsequent oligonucleotide mapping studies by Kennedy (1976) showed that both RNAs were derived from the 5' two-thirds and the terminal sequences of the 42S RNA.

In similar work, Pettersson (1981) serially passaged SFV in BHK-21 cells and after 11 passages isolated DI RNA species of about 2 kb from the cytoplasm of infected cells. This RNA was polyadenylated and oligonucleotide mapping showed that the 5'-terminal cap structures were heterogeneous and different from the 42S RNA. Subsequently, this DI RNA and one isolated from virus particles derived from the fourth passage were found to contain repeated sequences (Kääriäinen *et al.*, 1981). A more detailed analysis of the DI RNAs isolated by Pettersson was made through molecular cloning and sequencing of cDNA (Söderlund *et al.*, 1981; Lehtovaara *et al.*, 1981, 1982). Figure 1.4 shows the derivation of two of these RNAs (DI 301

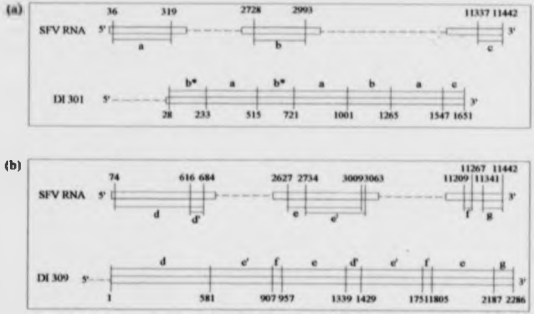


Figure 1.4 Comparison of DI 301 and DI 309 with SFV RNA

SFV RNA and the DI SFV RNAs (Lehtovaara *et al.*, 1981, 1982) are represented as linear sequence blocks. DI 301 and DI 309 have been simplified to show only extensive regions of homology with SFV RNA, which are labelled a-c in **(a)** and d-g in **(b)**. Because some regions of homology overlap, only the nucleotides at the 3' termini of each region of the DI RNAs are numbered. The dashed lines represent the nucleotide sequences at the 5' termini of the DI RNAs that have not been determined. In **(a)** the two regions labelled b* are deleted forms of region b, lacking the 60 nucleotides which correspond to nucleotides 2858 to 2917 of the SFV genome.

and DI 309) from SFV RNA, and has been updated to include the regions of SFV RNA which had not been sequenced at the time. The nucleotide sequences of DI 301 and DI 309 were determined from cDNA clones, both of which lacked the 5' termini (200-300 nucleotides) of the respective RNAs. The nucleotide sequence just within the 5' terminus of DI 309 was determined from direct sequencing of primer-extended cDNA. DI 301 contains three copies of regions derived from the 5' terminal sequences and the nsP2 gene of SFV and a region derived from the 3' terminus of SFV RNA. DI 309 has a more complex structure than DI 301, although it was derived from similar regions of SFV RNA. In addition to the regions derived from the terminal sequences and the nsP2 gene of SFV there is a short sequence of 59 nucleotides derived from the upstream sequences of the SFV 3' untranslated region that is represented twice in DI 309 (labelled 'f ' in Figure 1.4b). Analysis of the termini of a number of other DI RNAs showed that all retained regions derived from the 3' terminus of SFV RNA.

By definition DI genomes must possess the information required for replication and for packaging into virions and the information for interference and amplification. To investigate the functional regions of DI 301, Jalenko and Söderlund (1985) cloned the internal regions comprising the repeating units into an SV40 vector. This clone (svDI 301) lacked about 200 nucleotides from the 5' terminus and 70 nucleotides from the 3' terminus of DI 301. svDI 301 RNA was expressed by infection of monkey kidney cells. Following superinfection with SFV the RNA was not replicated but the packaging efficiency of SFV RNA was reduced by about 50%. They concluded that the repeating units of DI 301, which derived from the 5'-terminal sequences and the nsP2 gene of SFV (regions 'a' and 'b' in Figure 1.4a),

were involved in encapsidation and the terminal sequences of DI 301 were required for replication

(ii) Sindbis virus

DI RNAs of Sindbis virus are similar in structure to those of SFV. Early studies showed that DI RNAs of 20-50% of the standard genome size were generated by high-multiplicity passaging of Sindbis virus in BHK or CEF cells (Johnston *et al*., 1975, Guild and Stollar, 1975, 1977, Guild *et al*., 1977, Dohner *et al*., 1979). These RNAs were positive-stranded, polyadenylated and hybridisation studies showed they were composed of Sindbis virus RNA.

DI RNAs of Sindbis virus were later characterised by molecular cloning and sequencing (Monroe *et al*., 1982, Monroe and Schlesinger, 1983, 1984). Figure 1.5a shows the derivation of one such DI RNA (SS01) from the standard genome. Like the structure of the DI SFV genomes described by Lehtovaara *et al* (1982), this DI RNA contains sequences derived mainly from the 5' terminus of the standard genome which are repeated and rearranged. One feature of this DI RNA and of other DI RNAs of Sindbis virus produced by serial passaging in CEF cells is a sequence covalently linked to the 5' terminus which is homologous to nucleotides 10-70 of a rat $tRNA^{asp}$ sequence, differing by only 2 nucleotides (Figure 1.5b). This sequence was not associated with DI RNAs produced after passaging in BHK cells (Monroe and Schlesinger, 1984). The significance of the colinear $tRNA^{asp}$ sequence is not known, but Tsiang *et al* (1985) showed that it did not confer a competitive advantage over DI RNAs without the sequence.

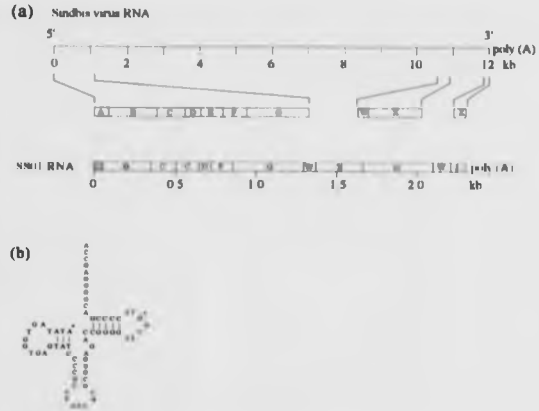


Figure 1.5 Structure of DI Sindbis virus RNA

(a) is reproduced from Monroe and Schlesinger (1984) and shows the derivation of the DI RNA of Sindbis virus (SS01) from the standard virus RNA SS01 RNA is represented as linear sequence blocks with the hatched area at the 5' terminus denoting cellular $tRNA^{asp}$

(b) is reproduced from Monroe and Schlesinger (1983) and shows the structure of the $tRNA^{asp}$ at the 5' terminus of two DI RNAs of Sindbis virus * denotes the two nucleotide differences with the rat $tRNA^{asp}$ sequence

In an attempt to define regulatory elements of Sindbis virus RNA, Levis *et al* (1986) deleted various regions of a clone of DI Sindbis virus and studied the effects this had on replication and packaging. They found that only 162 nucleotides derived from the 5' terminus and 19 nucleotides from the 3' terminus of the Sindbis virus genome were required for replication and packaging. These results were supported by Levis *et al* (1987) who replaced the internal sequences of a DI genome of Sindbis virus with the gene for chloramphenicol acetyl transferase (CAT). The termini of the DI genome supported the expression of CAT in infected cells and the recombinant genome was replicated and packaged by helper Sindbis virus. Further analyses by Tsiang *et al* (1988) showed that deletion of 11 nucleotides from the 5' terminus of a clone of DI Sindbis virus (corresponding to nucleotides 16-26 of Sindbis virus RNA) prevented transcripts from being propagated as virions. It was suspected that the stem-loop structure in this region, thought to be required for replication (Ou *et al*, 1983), had been disrupted.

Weiss *et al* (1989) used capsid protein-binding studies to identify a region of Sindbis virus RNA from nucleotides 746-1226 (within the nsP1 gene) that was required for encapsidation. A DI RNA that lacked this region was not detected on passaging, suggesting it was not packaged into virions. These results appear to conflict with the data provided by Levis *et al* (1986) who suggested that only the terminal sequences of the Sindbis virus genome were required for propagation of DI RNA as virions. The work of Weiss *et al* (1989) is probably more reliable because the assay used by Levis *et al* (1986) for analysing the effects of deletion did not distinguish between replication and propagation as virions. Furthermore, a clone containing only the terminal regions of the Sindbis virus genome was not

amplified on passaging, although it was suspected that this was too small to be packaged efficiently into virions

(iii) Other positive-strand RNA viruses

Apart from SFV and Sindbis virus, the only other positive-strand RNA viruses for which DI genomes have been characterised by sequencing are poliovirus and mouse hepatitis virus (MHV)

Early hybridisation studies on DI RNAs of poliovirus showed that only about 15% of the standard genome was deleted (Cole and Baltimore, 1973) In later studies, DI RNAs derived from the Sabin and Mohoney strains of poliovirus type 1 were shown to lack the genome region encoding the capsid protein (Nomoto *et al*., 1979, Kajigaya *et al*., 1985) Unlike the DI genomes of other positive-strand viruses, these retained the reading frame used by the standard virus genome (Kuge *et al*., 1986, Omata *et al*., 1986)

Recently, the DI genomes of MHV (a coronavirus) have been investigated Makino *et al* (1985) isolated a number of different DI MHV particles produced by high multiplicity passaging of MHV in tissue culture One type of DI RNA was almost genomic size and contained small multiple deletions throughout most of the standard genome The regions of the standard genome that encoded the polymerase gene, located at the 5' terminus, and a structural protein, located at the 3' terminus, were retained This DI RNA could replicate in the absence of helper virus and was weakly interfering (Makino *et al*., 1988) A second type of DI RNA of 2 3 kb was also isolated that retained the termini of the standard genome and a

region derived from the polymerase gene (gene 1). A similar DI RNA of 5.5 kb (DI-a) was isolated by van der Most *et al.* (1991). Unlike the DI genomes of SFV described by Lehtovaara *et al.* (1982), these did not possess sequence repeats or rearrangements. In addition, de Groot *et al.* (1992) showed that the three regions of DI-a which were derived from the termini and polymerase gene of the MHV genome were fused in-frame. Frameshift mutations reduced the accumulation of the DI RNA, which was ultimately competed out by mutant DI RNA with a restored reading frame.

DI RNAs of MHV, like those of Sindbis virus, have been used to identify regulatory elements within the standard genome. These studies have shown that the 3' terminus of the polymerase gene is required for encapsidation and the terminal regions of the standard virus genome for replication (Makino *et al.* 1990, van der Most, 1991).

(iv) Negative-strand RNA viruses

Of the DI genomes of negative-strand RNA viruses, those of VSV and influenza virus are the best studied, and are considered here because of their importance *in vivo*. Little is known about the DI genome structure of other negative-strand viruses.

The DI genomes of VSV are typically 10 to 60% of the standard genome length (reviewed in Perrault, 1981). A mixture of positive and negative sense DI RNAs may be packaged in the same particles, although the negative strands tend to be in excess in a given population (Roy *et al.*, 1973, Perrault and Leavitt, 1977).

Although it is difficult to generalise on the genome structure of DI viruses, sequencing studies of DI VSV have revealed two common types. Most DI VSV genomes have short-stem or panhandle structure, in which the 5' terminus of the standard genome is retained and the 3' terminus is replaced by sequences complementary to the 5' terminus. The stem of the panhandle structure can range from 45 to 200 nucleotides in length (Schubert *et al*., 1979, Kolakofsky, 1982). In the second common type of DI VSV genome the 3' half of the genome is entirely complementary to the 5' half and hairpin or 'snapback' structures are formed (Lazzarini *et al*., 1975, Perrault, 1976). Other rare DI VSV genomes have also been described which comprise single or multiple internal deletions and retain the termini of the standard genome (Kolakofsky, 1982, Kang *et al*., 1985, Rud *et al*., 1986).

About 5 DI genomes of influenza virus have now been analysed by sequencing and about 30 by hybridisation and oligonucleotide mapping studies (reviewed in Nayak *et al*., 1989). Unlike the DI genomes of VSV, those of influenza virus are derived solely from the negative-stranded RNA. In addition, the termini of the standard genome are retained and no snapback-type molecules have been found. Most DI genomes of influenza virus are derived from the polymerase genes by single or multiple deletion events. In addition, some DI genomes have internal sequences derived in part through intersegmental genome rearrangements (Lazzarini *et al*., 1981, Nayak *et al*., 1989).

(d) Generation and amplification of DI RNA

(i) Generation and evolution of DI RNA

Generation is defined as the initial event by which a DI genome is created. The precise mechanisms which produce DI genomes from the standard genome have not been determined but they presumably involve replication errors by the viral polymerase. The genome structures of DI virus permit speculation on such mechanisms, but this may be misleading because only those genomes that are amplified can be investigated. Studies of DI alphavirus during serial passage have shown that the genome size decreases with passage number (Johnston *et al*., 1975; Guild *et al*., 1977; Stark and Kennedy, 1978). This was thought to be a consequence of sequential deletion steps involving template jumping by the viral replicase, with smaller DI genomes being generated from larger ones. It was suspected that smaller DI genomes were replicated more frequently, thus competing out the larger DI genomes and becoming the predominant DI RNA species in the population (Kennedy, 1976; Stark and Kennedy, 1978). However, Kääriäinen *et al*. (1981) showed that the average size of DI SFV RNAs could increase on passage, presumably through duplication of parts of the genome. In addition, the sequence repeats and rearrangements of the DI SFV genomes described by Lehtovaara (1981, 1982) suggest a more complex mechanism for DI genome generation than a series of deletion steps.

Clearly the replicase complex of RNA viruses has a limited fidelity for replicating the standard genome. There is no evidence of preferred breakage points in the standard genome but it has been suggested that the replicase complex may be able

to copy non-contiguous regions of the template genomes which have become juxtaposed by RNA folding (reviewed in Perrault, 1981 and Holland, 1985). How repeated regions are incorporated into DI genomes is not known but it is possible that they derive from recombinational events between the DI genome and the standard or other DI genomes.

Makino and Lai (1989) suggested that the leader sequence of the MHV genome is involved in replication of DI RNA. They showed that following transfection of DI RNA into MHV-infected cells, the leader sequence of the DI RNA switched to the viral RNA after a single round of replication. They proposed that the generation of DI RNA may be triggered by the binding of free leader sequences derived from standard viral RNA to different sites within the template.

(ii) Amplification of DI RNA

Once a DI genome has been generated it can only become established in a virus population if it confers a selective advantage that enables it to be amplified relative to other viral RNAs. DI genomes are smaller than the standard virus genome and are presumably replicated more frequently in the infected cell as a consequence. However, this property alone does not explain the observations of Kääriäinen *et al* (1981) that smaller DI genomes can be competed out by larger ones. It is possible that amplification is achieved through the possession of multiple recognition sites for viral regulatory proteins, as described by Lehtovaara *et al* (1981, 1982) for DI SFV, but there is no evidence that such genomes are selected in preference to ones with only single recognition sites. It is likely that amplification is a multi-factorial process and the precise mechanisms involved remain to be determined.

(iii) Effect of host cell on DI RNA generation and amplification

Stark and Kennedy (1978) showed that DI SFV is generated and amplified at different rates in different cell types DI particles were produced after only a few passages of standard virus in mouse 3T3, rat NRK and CEF cells, whereas in HeLa-H cells, DI SFV was not detected after 200 passages However, DI virus could be propagated in all cell types In addition, Steacie and Eaton (1984) showed that some preparations of DI SFV generated in mosquito cells were not propagated in CEF cells Clearly the generation, amplification and propagation of DI viruses depends in part on host cell components In a number of HeLa cell types DI SFV is not propagated It has been suggested that in this situation, replication of viral RNA involves particular sequences that are recognised by the viral-host replicase complex and are absent from the DI genome (Dimmock, 1991) This implies that DI virus propagation in different cell lines is also dependent in part on the sequence of the DI genome

(e) Interference *in vitro*

By definition, interference results in a reduction in the yield of standard virus Interference is an intracellular event that involves an interaction of the DI genome with the replication cycle of the standard virus In addition, the degree of interference by a DI virus is greatest against the homologous virus from which it was derived (Huang, 1973, Barrett and Dimmock, 1984d)

Theoretically, interference can occur at any stage of the infectious cycle and the wide variety of DI genomes that have been isolated may be a reflection of this In

coinfected cultures, interference can be influenced by the host cell. It is dependent on the ratio of DI to standard virus in the inoculum and with excess standard virus no interference takes place (Perrault and Holland, 1972, Barrett *et al*., 1981, Debnath *et al*., 1991). In addition, DI virus preparations generated by serial undiluted passage are heterogeneous (Kääriäinen *et al*., 1981, Barrett *et al*., 1984a). For these reasons the resolution of the precise mechanisms involved in interference has proved difficult. However, a number of mechanisms have been proposed, some of which are described below.

(i) Alphaviruses

A number of studies have shown that coinfection of cell cultures with DI particles and standard virus results in a reduction in the levels of 42S and 26S viral RNAs (Weiss and Schlesinger, 1973, Johnston *et al*., 1975, Bruton *et al*., 1976, Guild *et al*., 1977). The synthesis of both positive and negative-strand RNA is inhibited and the total amounts of viral RNA reduced (Guild and Stollar, 1975, Barrett *et al*., 1981). In addition, a reduction in the number of cytopathic vacuoles associated with the viral replication complex has been observed (Levin *et al*., 1973). Interference by DI alphaviruses is therefore thought to occur at the level of RNA synthesis. Barrett and Dimmock (1985) showed that DI SFV could inhibit virus-specified RNA and polypeptide synthesis and inhibit the shut-off of host protein synthesis in coinfected cells. The specific effects on cellular and viral polypeptide synthesis differed according to the preparation of DI virus that was used. However, Jalenko and Söderlund (1985) showed that viral RNA synthesis was unaffected by a cloned preparation of DI RNA (svDI 301; see section 2b-4) and interference

occurred at the level of encapsidation. Clearly, different DI genomes of alphaviruses can interfere through different mechanisms.

Some DI alphavirus genomes can function as messenger RNAs in the infected cell, but there is no evidence for translation products being involved in interference (Weiss and Schlesinger, 1973; Guild and Stollar, 1975). In addition, some DI genomes do not have open reading frames and therefore must interfere solely at the level of the RNA (Lehtovaara *et al.*, 1981, 1982; Monroe and Schlesinger, 1984).

More generally, interference occurs through the competition for a limiting amount of standard viral nonstructural and structural proteins. The competitive ability of a DI genome may be enhanced by the possession of multiple recognition sites for regulatory proteins, which have been described for the DI genomes of SFV (Lehtovaara *et al.*, 1981, 1982) and some DNA viruses (reviewed in Perrault, 1981). However, there is no direct evidence that such repeated sequences can enhance levels of interference.

(ii) Negative-strand RNA viruses

A number of mechanisms have been proposed for interference by the different types of DI VSV genomes (see section 2b-*iv*, reviewed in Holland, 1985, 1990). In the short-stem genomes the 3'-terminal sequences of the standard viral RNA that contain the transcriptase recognition site are replaced by the 5'-terminal sequences of the standard RNA[1] containing the replicase initiation site. These genomes cannot therefore be transcribed into RNA[2] and like the snap-back DI genomes

possess replicase initiation sites in both RNA$^-$ and RNA$^+$ sequences. The replicative advantage of these genomes is suspected to be attributable to these features.

The DI genomes of influenza virus are generally derived by internal deletion of the standard genome. Some of these genomes are translated in the infected cell and it has been suggested that interference may be mediated in part through translation products, although there is no direct evidence for this (Penn and Mahy, 1985; Nayak *et al*., 1989).

(f) Modulation of infection *in vivo*

(i) Overview

A number of studies have shown that DI viruses can modulate infection *in vivo* after co-administration with standard virus. The degree of modulation can range from prevention of a normally lethal infection (protection) to a delay in the onset of disease signs. Modulation of infection *in vivo* by DI virus has been observed for a number of virus systems, the most extensively studied of which are VSV, SFV and influenza virus. In both the VSV and SFV systems, DI virus interferes with virus multiplication in the brain. In contrast, modulation of infection by DI influenza virus is mediated through cell-specific interactions with the immune system. DI influenza virus has no effect on virus multiplication in the lungs but interferes with standard virus in lymphocytes to prevent a lethal immune pathology (Dimmock *et al*., 1986; Morgan and Dimmock, 1992). It is therefore not pertinent to discuss the influenza virus system in detail here. For reviews on these and other DI virus

systems the reader should consult Barrett and Dimmock (1986), Dimmock (1991) and Roux *et al.* (1991).

(ii) VSV

Doyle and Holland (1973) first demonstrated that a normally lethal infection by intracerebral inoculation of adult mice with VSV could be prevented by administration of DI VSV. At least 10^{10} DI particles were required for protection and this was only effective against a small dose of standard virus. Further studies showed that protection was specific for homologous virus and was not a consequence of interferon induction (Holland and Doyle, 1973). While these studies failed to detect DI virus in the brains of protected adult mice, Holland and Villarreal (1975) indirectly demonstrated the presence of DI VSV in the brains of newborn mice by using an *in vitro* amplification step. In later studies, Cave *et al.* (1984, 1985) used northern blot analysis to detect DI VSV RNA in the brains of adult mice which had been inoculated by the intranasal route. For this, a nick-translated clone of a DI VSV genome was used as probe. These studies also showed that the ratio of DI to standard virus was an important determinant of protection. Protection was achieved after intranasal coinoculation of a 1:100 ratio of DI : standard virus while a 1:1 ratio did not protect.

The possibility that protection by DI VSV was immune-mediated was investigated by Crick and Brown (1977). They showed that acetyl ethyleneimine-inactivated DI VSV could protect mice against a lethal dose of VSV, suggesting that host responses were involved in protection. In addition, protection was observed against heterologous strains of VSV, rabies and a neurotropic strain of foot-and-mouth

disease. However, Jones and Holland (1980) and Fultz *et al.* (1982) showed that u.v.-irradiated DI VSV did not protect against a lethal dose of standard virus and protection therefore required a biologically active DI genome. These conflicting results may have been due in part to differences in the DI preparations and mouse strains that were used.

(iii) Semliki Forest virus

Dimmock and Kennedy (1978) first demonstrated that DI SFV generated in tissue culture could protect adult mice against a normally lethal dose of SFV inoculated by the intranasal route. The DI SFV preparation used typically conferred >50% protection when administered simultaneously with standard virus and reduced brain titres by 10^5-fold. In mice which did not survive, the infection followed its normal course. The greatest degree of protection against 10 LD_{50} (6,000 p.f.u.) was observed with a ratio of at least 67 'p.f.u.-equivalents' DI SFV to 1 p.f.u. standard virus. Protection was not mediated through interferon induction or stimulation of the host immune system and was suspected to be a consequence of intracellular interference by the DI genome with virus multiplication. Subsequent studies by Crouch *et al.* (1982) and Barrett *et al.* (1984c) demonstrated the complete absence of histopathological lesions in the brain tissue of protected mice. In addition, there was no evidence of immune cell infiltration, further suggesting that protection by DI SFV was not immune-mediated.

Barrett and Dimmock (1984c) described a number of factors important to the modulation of infection *in vivo* by DI SFV. Protection was influenced by the strain of mouse used, and was not observed in C_3H-He/Mg mice, although this may have

been a consequence of needing a higher titre of SFV for a lethal dose. The quantity of DI virus and SFV in the inoculum were also crucial to the protecting effect. Doubling the concentration of SFV or halving the concentration of DI virus resulted in a significant reduction in protection.

In subsequent studies by Barrett and associates, a comparison was drawn between two mouse-protecting DI SFV preparations (p13a and p4), which had been generated by serial undiluted passage of SFV in BHK-21 cells (p13a was derived from the preparation used by Dimmock and Kennedy, 1978). In all these experiments levels of SFV antigen were standardised by haemagglutination titres and u.v.-irradiated SFV was used to control for possible immunological effects and blockage of SFV receptors by DI virus. Following intranasal inoculation into adult mice, both p13a and p4 conferred about 60% protection against 10 LD_{50} SFV. However, mice protected with p13a were resistant to challenge with 100 LD_{50} SFV at 21 days after the initial inoculation and mice protected with p4 were totally susceptible (Barrett and Dimmock, 1984b; Barrett *et al*., 1984c). Neutralising antibodies in serum were detected in 75% of mice treated with p13a but only 30% of mice treated with p4. However, there was no evidence that the levels of circulating antibody were sufficient for protection. Following coinoculation with encephalomyocarditis virus (EMC), which is sensitive to interferon, no modulation of EMC infection was observed, suggesting that protection by DI SFV was not mediated through non-adaptive immune responses (Barrett and Dimmock, 1984b).

A comparison of the growth curves of SFV in mice treated with SFV, p13a or p4 was investigated by Barrett *et al* (1984c). Following intranasal inoculation of adult mice with SFV, virus was detected in the brain and olfactory lobes 1 day post-

infection (p.i.) and titres rose until death (4-5 days p.i.), reaching titres of 10^{10} p.f.u. per brain. In the spleen and serum, SFV was detected from day 2 and titres reached a peak of 10^8 p.f.u. per mouse. Following intranasal inoculation with either p13a or p4, SFV titres were reduced by >99% in a similar proportion of mice as expected from the lethality experiments. In p4-treated mice, the growth pattern of SFV in brain, olfactory lobes and spleen was similar to that of SFV-infected mice, but titres declined to low levels (10-100 p.f.u./mouse) from day 3. In p13a-treated mice, SFV titres were reduced compared with SFV-treated mice at all time points. In some mice the levels of infectivity in the spleen were similar to that of untreated mice but were reduced in other tissues. Notably, DI virus was not detected in any tissue of mice treated with either p13a or p4. It is possible that the assay used (an RNA synthesis inhibition assay), which detected $>10^{8.5}$ DI particles per ml, was not sufficiently sensitive.

Modulation of infection by p13a and p4 was also investigated after intraperitoneal inoculation (Barrett and Dimmock, 1984a). Both DI preparations conferred about 30% protection but a delay in death (36 hr) was observed after treatment with p13a.

In summary, the work of Barrett and associates showed that the modulation of infection *in vivo* by two different preparations of DI SFV differed in a number of respects, including susceptibility to challenge, tissue distribution of SFV and delay in death after intraperitoneal inoculation. These differences were not due to differences in SFV antigen levels or the degree of protection that was conferred. Furthermore, Barrett and Dimmock (1984b) showed that mouse-protecting ability of DI SFV preparations was not correlated with passage history. Sister stocks of

p13a and p4 conferred similar levels of protection, while daughter stocks differed in this respect, reflecting the heterogeneity of the preparations. It was concluded that the difference in interference properties of p13a and p4 was attributable to differences in their RNA sequences (Barrett *et al.*, 1984c).

Finally, the long-term effects of mouse-protection by DI SFV were investigated. Atkinson *et al.* (1986) showed that SFV could persist in the brains of mice treated with p4 or p13a. Virulent SFV was detected in 12% of p4-treated mice and 2.4% of p13a-treated mice 6.5 months after inoculation. In addition, Barrett *et al.* (1986) showed that in protected mice with no detectable infectious virus there were changes in the normal levels of certain neurotransmitters. The significance of these observations is not clear, but there may be implications for the aetiology of certain neurological disorders suspected to be associated with viruses.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

(a) Source of chemicals

All radioisotopes were obtained from Amersham International plc., Aylesbury, Bucks. All chemicals were of Analar grade, and were obtained from Sigma London Chemical Co. Ltd., Poole, Dorset, or BDH Chemicals Ltd., Poole, Dorset, unless otherwise stated. Nucleotide triphosphates (NTPs) and deoxynucleotide triphosphates (dNTPs) were obtained as 100mM aqueous solutions from Pharmacia Biosystems Ltd., Milton Keynes, U.K.

Tissue culture media and calf serum were purchased from Gibco BRL Europe Ltd., Paisley, Scotland. Components of bacterial media were obtained from Difco Laboratories Ltd., E. Molesey, Surrey.

(b) Source of enzymes

All enzymes were obtained from Gibco unless otherwise stated. Specific buffers for restriction enzymes, T4 DNA ligase and T3/T7 RNA polymerases were supplied with the enzymes. *Taq* DNA polymerase (AmpliTaq) was obtained from Perkin Elmer Cetus, Beaconsfield, Bucks.

METHODS

I. TISSUE CULTURE

(a) Culture of cells

BHK-21 clone 13-3P was obtained from the European Collection of Animal Cell Cultures (CAMR, Porton Down, Wiltshire, U.K.) BHK-21 cells were grown as monolayers in Glasgow's modification of Eagle's medium (GMEM, Eagle, 1959) supplemented with tryptose-phosphate broth (GMEM-BHK) HeLa cells were cultured in GMEM supplemented with non-essential amino acids (GMEM-NEAA) Cultures of primary chick embryo fibroblasts (CEF) were prepared as described by Morser *et al* (1973) and were grown in medium 199 For cell growth, media were supplemented with 5% (v/v) foetal calf serum (FCS) or new-born calf serum (NCS), 4mM glutamine, 100 Units/ml penicillin and 100 µg/ml streptomycin For more rapid growth, 10% serum was used All media and solutions used for cell culture were sterilised by membrane filtration through 0 2µm 500ml bottle filters (Costar UK Ltd , High Wycombe, Bucks)

Cells were incubated at 37°C in flat-bottomed plastic culture vessels (Nunclon (Gibco)) in an atmosphere of 5% CO_2 / 95% air

To prepare for sub-culturing, growth medium was removed by suction and cell monolayers were washed once with phosphate-buffered saline (PBS, pH 7 4) Cells were detached from the growth vessel with a 4 1 (v/v) mixture of versene (0 02% w/v in PBS) and trypsin (0 25% w/v in Tris-saline pH 7 7) at room temperature

Cells were pelleted by centrifugation at 200g for 5 min (4°C) and resuspended in 10ml growth medium by pipetting. Cells were counted using a Neubauer haemocytometer (Sigma) and seeded into fresh flasks.

(b) Storage of cells

Monolayers were grown to confluency. Following trypsinisation and centrifugation, cells were resuspended in culture medium containing 10% dimethyl sulphoxide (DMSO) and 15% FCS to a concentration of 3.5 x 10^6 cells/ml. Resuspended cells were aliquoted into freezing vials wrapped in cotton wool and cooled to -20°C over 2 hr with shaking every half hour. The cells were cooled to -70°C overnight and transferred to liquid nitrogen for storage.

(c) Virus preparation

For transfection experiments and interference assays, SFV derived from the full-length clone pSP6-SFV4 was used (Liljeström *et al*., 1991). This has similar pathogenic properties to the mouse-virulent L10 strain (Glasgow *et al*., 1991). For all other experiments, a plaque-purified stock of the virulent ts^+ strain of SFV was used (first described by Tan *et al*., 1969). This was originally obtained from the John Curtin School of Medical Research, Australia National University, Canberra.

SFV was propagated in BHK-21 or HeLa cell monolayers. Cultures were inoculated with a multiplicity of infection (m.o.i.) of 0.1 plaque-forming units (p.f.u.) per cell, and incubated for 18 hr at 37°C in culture medium containing 2% NCS. Tissue culture fluid was harvested and clarified by centrifugation at 2000g

for 10 min (4°C) and stored at -70°C. Stocks of SFV derived from the full-length clone pSP6-SFV4 were generated by transcription and transfection into BHK-21 cells, as described by Liljeström *et al.* (1991, see section 7c). The second passage after transfection (designated vSFV4-p2/F13) was used in DI SFV transfection experiments.

(d) Infectivity titration

SFV infectivity was determined by plaque assay. Virus was serially diluted in 10-fold steps in culture medium containing 2% NCS. 100µl samples were added centrally to just-confluent monolayers of BHK-21 cells in 5cm dishes (Nunclon (Gibco, U.K.)) and incubated at room temperature for 30 min. Plates were overlayed with a 1:1 mixture of 1% noble agar (2x GMEM-NEAA containing 5% NCS, 0.08% DEAE (diethylaminoethyl)-dextran, 200 U/ml penicillin and 200µg/ml streptomycin. Plates were incubated for 2 days at 37°C and live cells stained with 4 ml neutral red (0.01% (w/v) in PBS) for 2 hr at 37°C.

(e) Infectious centre assay

SFV-infected BHK-21 cells were serially diluted in 10-fold steps in GMEM-BHK containing 2% NCS. 100µl samples were added centrally to just-confluent monolayers of BHK-21 cells in 5cm dishes and adsorbed for 30 min at room temperature. Plates were overlayed, incubated and stained as for plaque assays (see section 1d).

(f) DI virus preparation

Cloned DI SFV was obtained by transfecting RNA transcribed from cloned DI DNA into SFV-infected BHK-21 monolayers (see section 7). This was subsequently passaged at a constant m.o.i. of 60 at 37° C. After 16 hr, sodium bicarbonate (5% (w/v) aqueous solution) was added drop-wise until the medium became pink in colour. Medium was harvested after 24 hr and clarified by centrifugation at 2000g for 10 min (4°C).

All other DI SFV preparations were generated previously by serial passage of the ts1 strain of SFV in BHK-21 cells at a constant m.o.i. of 50.

(g) Ultraviolet (u.v.) irradiation

Infectivity was removed from DI preparations by the method described by Dimmock and Kennedy (1978). 1 ml samples were placed in 5 cm dishes with occasional agitation 10cm below a u.v. lamp (254 nm, Gelman Sciences, Northampton, U.K.). One $\log_{10}$ p.f.u. was inactivated in 8s, as determined by plaque assay of u.v.-irradiated SFV. To determine loss of infectivity, samples were titrated on BHK-21 monolayers and cytopathic effect (c.p.e.) observed after 24hr.

(h) Treatment of DI preparations with β-propiolactone

To inactivate preparations of DI virus using β-propiolactone (BPL, grade II) the method described by Barrett *et al.* (1984d) was used. One tenth volume of 0.5M Tris.HCl (pH 7.5) was added to DI SFV in culture medium. 0.01% (v/v) BPL was

added and samples incubated on ice for 8 hr. To remove BPL, samples were dialysed overnight at 4°C against PBS

(i) Interference assay

The interfering activity of u.v - irradiated DI SFV was determined by making serial two-fold dilutions in 100µl medium containing 10 $TCID_{50}$ (50% tissue culture infectious dose) in a 96-well plate (Nunclon). BHK-21 cells (2×10^4 in 100µl) were added to each well, and plates incubated at 37°C for 24 hr. C.p.e. was noted at 24 hr.

2. NUCLEIC ACID PURIFICATION

(a) Phenol extraction

Phenol extraction was used to purify samples of nucleic acid. An equal volume of a 1:1 (v/v) mixture of phenol : chloroform was added to aqueous samples in Eppendorf tubes (Sarstedt, Walter U.K. Ltd., Leicester). 'Phenol' refers to phenol equilibrated with 0.1M Tris.HCl (pH 8.0) containing 0.1% hydroxyquinoline. 'Chloroform' refers to a 24:1 (v/v) mixture of chloroform : isoamyl alcohol. Tubes were mixed by vortexing and centrifuged for 15 sec at 15,000g. The aqueous layer was removed and the extraction process repeated until no pellet was visible at the interface. Finally, traces of phenol were removed by repeating the extraction once using an equal volume of chloroform.

(b) Ethanol precipitation

Aqueous samples of nucleic acid were concentrated by adding one-tenth volume of 3M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol. Samples were stored at -20°C for at least 30 min and centrifuged for 15 min at 15,000g. Pelleted nucleic acid was resuspended in sterile distilled water.

(c) Determination of nucleic acid concentration

Nucleic acid was quantitated using spectrophotometry. Samples were diluted in distilled water and transferred to quartz cuvettes. Optical density (OD) was measured at 260 and 280 nm. 1 OD_{260} was taken as equivalent to 50μg/ml for double-stranded DNA, 40μg/ml for single-stranded RNA or 20μg/ml for single-stranded DNA. The ratio of nucleic acid to protein was determined from OD_{260} divided by OD_{280} and was used as a measure of purity. For pure preparations, this ratio was 1.8 (DNA) or 2.0 (RNA).

3. RNA ANALYSIS

(a) Extraction of RNA from tissue culture cells

Total cellular RNA was extracted from tissue culture cells using a modification of the method described by Gilfin *et al* (1974). Monolayers of BHK-21 cells were treated for 20 min at room temperature with 4M guanidinium isothiocyanate in 10mM Tris.HCl (pH 7.5). CsCl was added to a final concentration of 0.15g/ml and N-lauroyl-sarcosine to 0.4% (w/v). Homogenates were layered on one-fifth volume

5.7M CsCl containing 0.1M EDTA (ethylenediamine tetraacetic acid, pH 7.0) and centrifuged at 100,000g for 20 hr at 20°C. Pelleted RNA was resuspended in 10mM Tris.HCl (pH 8.0), 1mM EDTA, 0.1% sodium dodecyl sulphate (SDS), extracted with phenol and ethanol-precipitated. Purified RNA was resuspended in sterile distilled water and stored at -70°C. Typically 1μg RNA was obtained from 10^6 cells.

(b) Extraction of RNA from brain tissue

Adult mice were killed with ether and dissected brains stored at -70°C. Thawed brains were suspended in guanidinium isothiocyanate lysis buffer (see section 3a) and homogenised through a 19-gauge hypodermic syringe needle (Becton-Dickinson U.K. Ltd., Oxford). Homogenates were treated as described in section 3a. Typically, 100μg RNA were obtained from one brain.

(c) Extraction of RNA from tissue culture fluid

RNA was extracted from small volumes (typically 75μl) of tissue culture fluid with an equal volume of 10 mM Tris.HCl (pH 7.9), 350 mM NaCl, 10mM EDTA, 7M urea, 1% (w/v) SDS followed by phenol extraction and ethanol precipitation (after A. J. Easton, personal communication). RNA was resuspended in distilled water and stored at -20°C. For analysis by RT-PCR, RNA was resuspended directly in reaction buffer.

For larger volumes, virus was pelleted by centrifugation at 100,000g for 3 hr at 4°C and resuspended in TNE (0.1M NaCl, 10mM Tris.HCl pH 7.5, 1mM EDTA

pH 8.0) Proteinase K was added to 200µg/ml and SDS to 0.1%. Samples were incubated at 37°C for 30 min and RNA extracted with phenol. RNA was concentrated by ethanol-precipitation and resuspended in distilled water and stored at -70°C.

(d) Analysis of RNA by agarose gel electrophoresis

RNA was resolved on agarose gels after denaturation with glyoxal and dimethyl sulphoxide (after McMaster and Carmichael, 1977). 5.4µl of an aqueous solution containing up to 20µg RNA was mixed with 5.4µl of 6M glyoxal, 16µl DMSO and 3.2µl 0.1M Na_2HPO_4 (pH 7.0). The mixture was incubated at 50°C for 60 min and 3.0µl RNA loading buffer added (50% glycerol, 0.01M Na_2HPO_4, 0.4% bromophenol blue). A 1.0% horizontal agarose gel was prepared as described in section 4a, but using 0.01M Na_2HPO_4 as gel and running buffer and no ethidium bromide. Samples were electrophoresed for 2 hr at 4V/cm. Buffer was circulated from the anode to the cathode using a peristaltic pump. Following electrophoresis, RNA was stained for 30 min with 10µg per ml ethidium bromide and the gel was subsequently de-stained for 60 min in running buffer. Stained RNA was visualised on a u.v. transilluminator and photographed as for DNA (see section 4a). Molecular size was determined by comparison to RNA size markers (RNA ladder Gibco).

(e) Northern blot analysis

To analyse SFV RNA by northern blotting, samples were firstly resolved on an agarose gel following denaturation with glyoxal (see section 3d). After

electrophoresis, only the RNA ladder was stained with ethidium bromide, and the test samples were transferred to a hybridisation filter (after Southern, 1975). Two strips of Whatman 3MM filter paper (Whatman Labs Sales Ltd., Maidstone, Kent) were placed on a glass support above a reservoir of 7.5mM NaOH, with the ends of the paper submerged in the buffer. The gel was placed on the filter paper, and a nylon filter (Hybond N Amersham) on the gel. Two squares of Whatman 3MM paper were placed on the nylon filter and paper towels on these. A 500g weight was placed on the paper towels and transfer allowed to proceed overnight.

After transfer, nylon filters were submerged in 30 ml hybridisation buffer (see below) for 4 hr at 42°C in a plastic box with constant shaking. For hybridisation, filters were transferred to 10-15 ml fresh hybridisation buffer pre-heated to 42°C and the probe added (see section 3g). Following incubation at 42°C for 16 hr, unbound probe was removed by washing the filter for 10 min at room temperature with 50ml 6 x SSC + 0.5% SDS, 10 min at 37°C with 50 ml 2 x SSC + 0.1% SDS, 20 min at 42°C with 50 ml 1 x SSC and 30 min at 60°C with 50 ml 1 x SSC + 0.1% SDS. Filters were wrapped in clingfilm to prevent dehydration.

Washed filters were transferred to an autoradiography cassette with X-ray film (Fuji (U.K.) Photo Film Co., London) and stored at -70°C for 16 hr. Film was submerged in developer fluid (Kodak Photographic Ltd., Hemel Hempstead, Herts.) for 3 min and transferred to fixative (Kodak) for 3 min. Finally, films were washed in tap water and dried. Where necessary, autoradiography was repeated for a longer or shorter period of time.

Note buffers used were as follows -

Hybridisation buffer	6x SSC, 10x Denhardt's solution, 50µg/ml herring sperm DNA (boiled for 10 min before use)
1x SSC	1.5 M NaCl, 0.015 M sodium citrate pH 7.0
100x Denhardt's solution	20g/l Ficoll, 20g/l polyvinylpyrrolidone, 20g/l bovine serum albumin (BSA, fraction V)

(f) Dot blot analysis

100 µl RNA samples in TE (10mM Tris HCl pH 8.0, 1mM EDTA pH 8.0) were mixed with an equal volume of formamide and incubated at 50°C for 15 min. Samples were transferred to Hybond-N filters by applying under vacuum to a Hybri-blot apparatus (Gibco). Each well was washed through with 100µl TE (pH 8.0). The filters were dried on Whatman 3MM paper and RNA fixed by u.v. by placing on a transilluminator (254 nm) for 4 min. RNA was analysed using labelled probes as described in section 3e. Autoradiographs were subsequently analysed using a scanning densitometer (Molecular Dynamics Ltd., Sevenoaks, Kent).

(g) Hybridisation probes

Oligonucleotide probes were made by end-labelling synthetic primers (see section 3h). 1µl primer (0.2 mg/ml) was mixed with 1µl 10x linker kinase buffer, 20µCi [γ-^{32}P]ATP, 20 U T4 polynucleotide kinase and diluted to 10µl with distilled water. The mixture was incubated at 37°C for 30 min and the reaction stopped by adding 2µl 0.5M EDTA (pH 8.0).

Nick-translated probes were made using cloned DI SFV (see Chapter 4) 1µg DNA was mixed with 5µl 10x nick translation buffer, 1µl 1mM each dNTP, 20µCi [α-^{32}P]dNTP and distilled water to 44µl. The mixture was chilled on ice for 5 min and 0.5µl DNAse I (0.1 µg/ml) added. 5 U *E.coli* DNA polymerase I was added, and the mixture incubated at 16°C for 60 min. The reaction was stopped by adding 2µl 0.5M EDTA (pH 8.0).

RNA probes were made by transcribing cloned DI SFV (see Chapter 4). Transcription was performed as described in section 7a except that 20µCi [α-^{32}P]UTP was included in the reaction.

After probes were labelled, unincorporated label was removed. Samples were diluted to 50µl with TE (pH 8.0) and loaded on a 5ml Sephadex G50 (Pharmacia) column equilibrated in TE (pH 8.0). 30 100µl fractions were collected and analysed with a Geiger-Muller counter (Mini-Instruments Ltd., Burnham-on-sea, Essex). The six fractions with the highest readings and comprising the first peak of radioactivity (usually between fractions 10 and 20) were pooled and stored at -20°C.

Note: buffers used were as follows -

10x linker kinase buffer	0.66 M Tris HCl (pH 7.6), 10mM spermidine, 0.1 M $MgCl_2$, 150 mM dithiothreitol (DTT), 2 mg/ml BSA
10x nick translation buffer	0.5 M Tris HCl (pH 7.2), 0.1 M $MgSO_4$, 1mM DTT, 500µg/ml BSA

(h) Reverse transcription and polymerase chain reaction (RT-PCR)

The polymerase chain reaction (PCR) was used to amplify cDNA templates (after Saiki *et al*., 1985). RNA was reverse transcribed and amplified by PCR in 100µl reaction buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 0.01% gelatin, 4.5mM $MgCl_2$, 0.5mM each dNTP, 2 U AMV reverse transcriptase (Pharmacia), 1.5 U *Taq* polymerase, 20 pmol each primer), and overlayed with 100µl paraffin. For analysis of DI SFV RNA, the primers *5'SFV* and *3'SFV* were used, which were homologous to the 5' terminus and complementary to the 3' terminus of SFV RNA respectively. For production of DNA for cloning, the promoter for T3 RNA polymerase was incorporated on the 5' primer (*5' SFV-T3*), and the restriction enzyme site for NcoI on the 3' primer (*3' SFV-Nco*). For analysis of SFV RNA the primers *SFV-5i* and *SFV-3i* were used, which anneal within the nsP2 gene of SFV.

Reactions were performed using a thermal cycler (Techne (Cambridge) Ltd., or Hybaid Ltd., Teddington, Middlesex). Primer annealing temperatures were determined from an estimate of their melting temperatures (T_m see Chapter 3). Typical reaction times were 40 min at 42°C for reverse transcription, followed by 30 cycles of 60 s at 94°C, 60 s at (T_m-2°C) and 80 s at 72°C. A final step of 5 min at 72°C was included before cooling to 20°C. PCR products were analysed by agarose gel electrophoresis (see below) and were stored at -20°C. RT-PCR using the primers *5'SFV-T3* and *3'SFV-Nco* was performed using an annealing temperature of 54°C.

Primers *

5'SP1 (bases 1-25) ..... 5'-ATG GCG GAT GTG TGA CAT ACA CGA C-3'

3'SP1 (bases 11442-11411) ..... 5'-GGA AAT ATT AAA AAC CAA TTG CAA AAT AAA AAT-3'

5' SFV-T3 ..... |----------*T3*----------| 5'-GCA ATT AAC CCT CAC TAA AGA TGG CGG ATG TGT GAC ATA CA-3'

3' SFV-Nco. ..... |—*NcoI*—| 5'-TTC CAT GGG AAA TAT TAA AAA CCA ATT GCA-3'

SFV-5i (bases 1780-1798) ..... 5'-CCC GCA GAC CGT GCT CAA G-3'

SFV-3i (bases 2512-2495) ..... 5'-CCG AGG TTT AAC AAG AGC-3'

* Numbers refer to nucleotides of SFV RNA. Primers were made with a DNA synthesiser (3 column, Applied Biosystems, Warrington, Lancs.)

(i) In vitro *translation*

Translation of RNA transcripts was performed using a rabbit reticulocyte lysate translation system (Gibco), and the protocol and reagents supplied with the kit. Following transcription (see section 7a), the reaction mixture was extracted with phenol and ethanol-precipitated. RNA was resuspended in 50µl DEPC (diethyl pyrocarbonate)-treated water. 0.5 or 1.0µl RNA were mixed with 3µl 10x translation mixture, 2.4µl 1M potassium acetate, 25µCi [^{35}S]-methionine, 10µl rabbit reticulocyte lysate and DEPC-treated water to 30µl. The mixture was incubated at 30°C for 60 min and cooled on ice.

10µl reaction mixture was mixed with 10µl protein sample buffer and incubated at 100°C for 2 min. Denatured translation products were electrophoresed on vertical 10-20% gradient SDS-polyacrylamide gels (BioRad Laboratories Inc., Hemel Hempstead, Herts.) at 10V/cm in electrode buffer, after the method of Laemmli (1970). For size determination, an unlabelled Rainbow marker was used (Amersham). Gels were analysed by autoradiography as described in section 3e.

Note: buffers used for gel electrophoresis were as follows -

Protein sample buffer: 3.2ml 5M urea, 1.0ml Tris.HCl (pH 6.8), 0.8ml glycerol, 1.6ml 10% SDS, 0.7ml 0.5% bromophenol blue, 1.2ml distilled water

5x electrode buffer: 94g/l glycine, 15.1g/l Tris base, 5g/l SDS

4. DNA ANALYSIS

(a) Agarose gel electrophoresis

DNA samples were mixed with one-fifth volume marker dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% glycerol). Samples were loaded on horizontal agarose gels (1-2% (w/v) agarose type II, medium EEO) submerged in TAE buffer (0.04M Tris-acetate, 0.001M EDTA pH 8.0). 50µg/ml ethidium bromide was included in the gel. Electrophoresis was performed at 1-4V/cm until the marker dye reached two-thirds distance along the gel.

DNA was visualised by placing the gel on a u.v. transilluminator (UV products Ltd., Cambridge) and was photographed with black and white film (type 55, Polaroid UK Ltd., St Albans, Herts.)

(b) Restriction enzyme analysis

Up to 5µg DNA in distilled water or TE (pH 8.0) was mixed with 1.5µl of the appropriate 10x buffer, 5 Units restriction enzyme and distilled water to 15µl. Samples were incubated at 37°C for 1-2 hr and analysed by agarose gel electrophoresis.

(c) Purification of DNA resolved on agarose gels

DNA fragments resolved on agarose gels were purified using Geneclean II® (BIO 101 Inc. (Stratech Scientific), Luton, Beds.) and a modification of the instructions supplied with the kit (derived from Vogelstein and Gillespie, 1979). Agarose gel containing the DNA of interest was excised using a scalpel and added to 3 volumes 6M NaI. Agarose was dissolved by incubating at 55°C for 5 min. 5µl Glassmilk was added and the suspension mixed on ice for 10 min. Samples were centrifuged at 15,000g for 5 s and the supernatant discarded. The pellet was washed three times with NEW wash (based on NaCl, ethanol, water) and resuspended in 50µl distilled water. Samples were incubated at 55°C for 3 min and centrifuged at 15,000g for 30 s. The supernatant was ethanol-precipitated and purified DNA resuspended in the desired volume of distilled water.

(d) Southern blot analysis

DNA was resolved on agarose gels and transferred to nylon filters by a modification of the method described by Southern (1975). Following electrophoresis, gels were soaked in 1.5M NaCl and 0.5M NaOH for 1 hr at room temperature. The gel was neutralised in 1M Tris HCl (pH 8.0) and 1.5M NaCl for 1 hr at room temperature with constant shaking. DNA was transferred from the gel to Hybond N filters as described in section 3e, but using 10x SSC (see section 3e) as transfer buffer. Following overnight transfer, DNA was fixed by placing the filter on a u.v. transilluminator (254 nm) for 4 min. Hybridisation and autoradiography was performed as described in section 3e.

5. MOLECULAR CLONING

(a) Cell culture

For growing pSP6-SFV4 (see section 5c), *Escherichia coli* (*E. coli*) strain DH5α was used (Stratagene Ltd., Cambridge). For all other plasmids, *E. coli* strain TG2 was used (obtained from Dr A. J. Easton).

TG2 cells were grown in LB (Luria-Bertani) medium (10g/l Bacto-tryptone, 5g/l Bacto-yeast extract, 10g NaCl, pH 7.5). DH5α cells were grown in SOB (20g/l Bacto-tryptone, 5g/l Bacto-yeast extract, 0.5g/l NaCl, 2.5mM KCl, 10mM $MgCl_2$). For solid medium, LB-medium was supplemented with 1.5% (w/v) Bacto-agar (LB-agar). All media were sterilised by autoclaving.

To culture cells harbouring plasmids, media were cooled to 37°C after autoclaving and ampicillin added (sterilised through a 0.2µm filter, Sartorius Ltd., Epsom, Surrey). For liquid media, 50µg/ml ampicillin were used, and 100µg/ml ampicillin for solid media.

Liquid cultures were grown at 37°C and 200 rpm in an orbital incubator (NBS Ltd., Hatfield, Herts.). For long-term storage, a single bacterial colony was inoculated in 5ml medium and grown overnight. 0.85ml was mixed with 0.15ml glycerol and transferred to a freezing vial. Cells were stored at -70°C.

(b) Preparation of PCR products for cloning

PCR products for subsequent cloning were purified, phosphorylated and end-filled. DNA derived from 100µl PCR reaction mixture was purified by phenol extraction, concentrated by ethanol precipitation and resuspended in 20µl distilled water. This was mixed with 20µl linker kinase buffer (see section 3g), 1µl ATP (100mM), 20 U T4 polynucleotide kinase and distilled water to 100µl. Samples were incubated at 37°C for 30 min, extracted with phenol and ethanol-precipitated.

DNA was resuspended in 20µl distilled water and mixed with 2µl 2mM 4x dNTPs, 5µl nick-translation buffer (see section 3g), 12 U large fragment of *E.coli* DNA polymerase I and distilled water to 50µl. Samples were incubated at 20°C for 30 min, phenol-extracted and ethanol-precipitated. Finally, DNA was resuspended in 30µl distilled water. 10µl was analysed by agarose gel electrophoresis, 10µl was stored at -20°C and 10µl used for ligation.

(c) Preparation of vector DNA for cloning

The full-length SFV clone pSP6-SFV4 was obtained from Professor P Liljestrom, Department of Molecular Biology, Karolinska Institute, Huddinge, Sweden

pBluescribe KS$^+$ (*amp*^{R}) was obtained from Stratagene pUC13 (*amp*^{R}, Yanisch-Perran *et al*, 1985) was obtained from Dr A J Easton Both vectors have polylinkers within the gene for β-galactosidase permitting blue-white selection of recombinants To prepare vectors for ligation, 1μg plasmid DNA was digested with the appropriate restriction enzyme, phenol-extracted and ethanol-precipitated DNA was resuspended in 20μl distilled water and stored at -20°C For blunt-ended ligations, the restriction enzyme was SmaI

(d) Ligation

For blunt-end ligating modified PCR products into plasmid vectors, 10μl sample was mixed with 5μl SmaI-digested plasmid DNA (40μg/ml), 4μl ligase buffer and 1 U T4 DNA ligase Samples were incubated overnight at 16°C and stored at -20°C

(e) Preparation of competent cells and transformation

A modification of the method described by Mandel and Higa (1970) was used A loopful of bacterial culture was inoculated in 5ml medium and incubated at 37°C overnight 2ml culture was diluted in 100ml medium in a conical flask and incubated at 37°C and 200 rpm until the OD_{450} of the culture reached 0 3 to 0 5

(usually 2 hr). Cells were collected in Oakridge tubes (Techmate Ltd., Milton Keynes) and chilled on ice for 10 min. Tubes were centrifuged at 1500g for 4 min (4°C) in a swing-out rotor and the supernatant discarded. Cells were resuspended on ice in 10ml ice-cold 100mM $MgCl_2$ and centrifuged as above. The process was repeated using 5ml ice-cold 100mM $CaCl_2$ (the dihydrate form) and finally 1.5ml 100mM $CaCl_2$. Cells were incubated for 1 to 12 hr on ice, after which they were considered to be competent to take up DNA.

200µl competent cells were added to 10µl ligation mixture. Cells were incubated on ice for 30 min with shaking. Tubes were transferred to a 42°C water bath for 90 s and returned to ice for 5 min. 200µl 2x LB medium were added and the cells incubated at 37°C, 200 rpm for 30 min.

For blue-white selection of colonies, transformed cells were inoculated on LB-agar containing 50µg/ml ampicillin, 0.005% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 0.1mM IPTG (isopropylthiogalactoside) in 9 cm plastic plates. Plates were incubated at 37°C overnight, and white colonies selected for further analysis.

(f) Small-scale plasmid preparation

A modification of the method described by Holmes and Quigley (1981) was used. Bacteria from a single colony were inoculated in 5ml L-broth containing 50µg/ml ampicillin. Following overnight incubation at 37°C in an orbital incubator, 1.5ml samples were taken and centrifuged at 15,000g. Bacterial pellets were resuspended in 350µl STET (8% (w/v) sucrose, 0.5% (v/v) Triton-X-100, 50mM EDTA pH

8.0, 10mM Tris HCl pH 8.0). 20µl fresh lysozyme (12 mg/ml) was added and the samples mixed by vortexing. Samples were incubated in a boiling water bath for 40 s and centrifuged at 15,000g for 10 min. The pellet was removed with a toothpick and 40µl 2.5M sodium acetate and 420µl isopropanol added to the supernatant. Samples were stored at room temperature for 5 min and centrifuged at 15,000g for 10 min (20°C). Pellets were rinsed with 70% ethanol and resuspended in 100µl TE (pH 8.0). Samples were treated with 10µl RNAse A (50µg/ml) for 10 min at 37°C and phenol-extracted. Plasmid DNA was concentrated by ethanol precipitation and resuspended in 20µl distilled water.

(g) Large-scale plasmid preparation

Plasmid DNA was extracted from large-scale bacterial cultures using a modification of the method described by Birnboim and Doly (1979). A loopful of a bacterial culture harbouring the plasmid of interest was inoculated into 500ml LB medium (*E.coli* TG2) or SOB (*E.coli* DH5α). This was placed in an orbital incubator at 37°C for 16 hr. Bacteria were harvested by centrifugation at 2000g for 45 min, resuspended in 8ml Solution I and transferred to an Oakridge tube. 1ml fresh lysozyme (10 mg/ml in 10mM Tris HCl pH 8.0) and 10ml freshly made Solution II was added, and the tubes mixed thoroughly by inversion. After 10 min at room temperature, 12ml ice-cold Solution III was added and the tubes mixed by inversion and stored on ice for 10 min. Tubes were centrifuged at 10,000g for 30 min and the supernatant filtered through 3 layers of muslin. 0.6 volumes of isopropanol were mixed with the supernatant and the tubes stored at room temperature for 15 min. To pellet nucleic acids, the tubes were centrifuged at

1500g for 15 min (20°C) The pellet was rinsed in 70% ethanol, dried by inverting the tubes on paper towels and resuspended in 3 ml TE (pH 8.0)

To purify plasmid DNA, the method described by Maniatis *et al* (1989) was used. 3 ml ice-cold 5M LiCl was added to the nucleic acid solution and the mixture centrifuged at 5,000g for 15 min (4°C) An equal volume of isopropanol was mixed with the supernatant and the solution centrifuged at 5,000g for 15 min (20°C) The pellet was rinsed with 70% ethanol and resuspended in 500µl TE (pH 8.0) containing 20µg/ml RNAseA After 30 min at room temperature, the sample was mixed with 500µl 1.6M NaCl containing 13% (w/v) polyethylene glycol 6000 and centrifuged at 15,000g for 5 min The pellet was dissolved in 400µl TE (pH 8.0) and phenol-extracted 100µl 10M ammonium acetate and 1ml ethanol were added and the samples stored at room temperature for 10 min Plasmid DNA was pelleted by centrifugation at 15,000g for 5 min, rinsed with 200µl 70% ethanol and dissolved in 500µl TE (pH 8.0)

Note buffers used were as follows -

Solution I 50mM glucose, 25mM Tris HCl (pH 8.0), 10mM EDTA

Solution II 0.2N NaOH, 1% (w/v) SDS

Solution III 60 ml 5M potassium acetate + 11.5 ml glacial acetic acid + 28.5 ml distilled water

6. DNA SEQUENCING

(a) Preparation of plasmid DNA

Plasmid DNA was prepared as described in section 5f. For sequencing, 4μg DNA in 5μl distilled water was used. To prepare single-stranded DNA, 15μl 0.27M NaOH + 0.27mM EDTA was added and the mixture incubated at room temperature for 5 min. DNA was precipitated by adding 2μl 3M sodium acetate (pH 4.6) and 40μl 95% ethanol. The mixture was incubated at -20°C for 10 min and centrifuged at 15,000g for 15 min. The pellet was washed once with 100μl 70% ethanol and centrifuged for a further 2 min. The pellet was rinsed with 95% ethanol and dried in a vacuum desiccator for 10 min.

(b) Sequencing using the chain termination method

Single-stranded plasmid DNA was sequenced using Sequenase® version 2.0 (USB (Cambridge Bioscience), Cambridge) and the protocol supplied with the kit (after Sanger *et al*., 1977). The primers used are listed in Figures 5.1 and 5.6. All buffers and reagents except label were supplied with the kit.

A mixture was prepared of 2μl reaction buffer, 2μl primer (0.5 pmol/μl) and 6μl distilled water. This was added to the dried plasmid preparation and incubated at 65°C for 2 min. The sample was cooled to 20°C over a period of 30 min and stored on ice.

For the labelling reaction, the sample was mixed with 1µl 0 1M DTT, 2µl labelling mix (diluted 1:5 in distilled water), 10µCi [^{35}S]-dATP 2 5µl each of the four dideoxy-NTPs were pre-heated to 37°C in separate Eppendorf tubes 2µl Sequenase 2 0 (diluted 1 8 in enzyme dilution buffer) was added to the DNA sample and incubated at room temperature for 3 min 4 x 3 5µl were added to the sides of the tubes containing the ddNTPs and the tubes centrifuged briefly to start the reactions The tubes were incubated at 37°C for 5 min 4µl stop solution was placed on the side of each tube and mixed to stop the reactions by brief centrifugation Samples were stored at -20°C

(c) Cycle sequencing

Some regions of cloned DNA were sequenced using a thermal cycling method, for reasons stated in Chapter 5 For this a Δ*Taq* kit (USB) was used and a modification of the protocol supplied with the kit All buffers and reagents except label were supplied with the kit

Double-stranded plasmid DNA was labelled by mixing 5µg with 0 5µl linker kinase buffer (see section 3g), 20µCi [α-^{32}P]ATP, 20 U T4 polynucleotide kinase and distilled water to 5µl Samples were incubated at 37°C for 30 min

Labelled DNA was diluted to 9 5µl with distilled water and mixed with 2µl reaction buffer, 1µl primer (0 5 pmol/µl) and 2µl *Taq* polymerase (diluted 1 8 in enzyme dilution buffer) 4 x 3 8µl of the reaction mixture was added to 3 8µl of each of the four ddNTPs and the samples overlayed with 8µl paraffin

Samples were incubated on a thermal cycler (Hybaid) and subjected to 15 cycles of 94°C for 60 s, 60°C for 60 s and 72°C for 80 s. Samples were cooled to room temperature and 4µl stop solution added.

(d) Analysis of products from sequencing reactions

Products from sequencing reactions were analysed on vertical polyacrylamide gels. Gels were prepared by mixing 18.6ml 40% acrylamide:N-N'-methylene bisacrylamide (19:1 solution, Severn Biotech Ltd., Kidderminster, Glouca.) with 63g urea, 15 ml 10x TBE (0.89M Tris-borate, 0.89M boric acid, 0.02 EDTA) and distilled water to 150 ml. Once the urea had dissolved, the solution was filtered and 150µl TEMED (N,N,N',N'-tetramethylenediamine) and 0.84 ml 10% (w/v) fresh ammonium persulphate was added. The mixture was poured between taped glass sequencing plates, combs inserted, and the gel set overnight.

Gels were placed in a vertical sequencing apparatus (Gibco) with TBE as buffer. Gels were pre-run at 80W for 30 min. Samples were incubated at 80°C for 2 min, loaded on the gel and electrophoresed at 80W for up to 4 hr. Plates were dismantled and fixed in 10% (v/v) glacial acetic acid for 45 min. After transfer to Whatman 3MM paper, gels were dried at 80°C for 1 hr using a vacuum drier. Autoradiography was performed as described in section 3e.

(e) Analysis of nucleotide sequences

Nucleotide sequences were stored and analysed using a microgenie database. For secondary structure predictions, data was analysed using a GCG software package (University of Wisconsin, Madison, USA).

7. TRANSFECTION OF RNA INTO TISSUE CULTURE CELLS

(a) RNA transcription

For production of RNA transcripts, 1µg plasmid DNA was mixed with 5µl 100mM DTT, 2.5µl BSA (2.5 mg/ml), 4 x 2.5µl each 20mM NTP, 10µl 10x transcription buffer, 25 U human placental RNAse inhibitor, 25 U RNA polymerase (T3 or T7) and distilled water to 50µl. For production of capped transcripts, 10µl 5mM sodium $m^7G(5')pppG$ (Pharmacia) was included in the reaction and the concentration of GTP halved.

The reaction mixture was incubated at 37°C for 1 hr. To remove template, 5 U RNAse-free DNAse (Promega Ltd., Southampton, Hants.) was added and the mixture incubated for a further 10 min. Size and integrity of RNA was determined on agarose gels (see Chapter 6). For transfection experiments, RNA transcripts were used on the day of preparation.

(b) Hypertonic shock

In preliminary experiments, the method of hypertonic shock was used to transfect RNA transcripts into tissue culture cells. CEF or BHK-21 cells were grown as monolayers in 5 cm dishes. Medium was removed by suction and the monolayers washed at room temperature with unbuffered saline.

20µl RNA transcription reaction mixture was diluted with 180µl 1.5M NaCl, 10mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, pH 7.2) and this was inoculated centrally on the monolayer. After incubation at room temperature for 10 min, warm medium was added and cells incubated for 10 min at room temperature. Medium was removed and 10 p.f.u./cell SFV in 3 ml medium/plate added. Cells were incubated at 37°C for 24 hr. Tissue culture fluid was harvested and passaged as described in section 1d.

(c) Electroporation

In later experiments, RNA transcripts were transfected into BHK-21 cells using a method adapted from Liljeström *et al.* (1991).

BHK-21 cell monolayers were incubated with 10 p.f.u./cell SFV in GMEM-BHK at room temperature for 45 min. Monolayers were trypsinised and centrifuged as described in section 1a. Cells were resuspended in 10 ml GMEM-BHK and centrifuged at 200*g* for 5 min. This process was repeated with ice-cold PBS and cells were finally resuspended in PBS (20°C) at a concentration of 10^7 cells/ml.

0 5 ml cell suspension was transferred to an electroporation cuvette and mixed by pipetting with 20µl RNA transcription reaction (approximately 20µg RNA see Chapter 6) After the addition of RNA, cells were immediately subjected to 2 consecutive pulses at 1 5kV/25µF using a Gene Pulser apparatus (BioRad), with the pulse controller unit set at maximum resistance

Cells were diluted in 5 5 ml GMEM-BHK and 2 x 3 ml transferred to 5 cm dishes Plates were incubated at 37°C for 18 hr Culture medium was harvested and clarified by centrifugation at 1500g for 10 min (4°C) Medium was passaged as described in section 1d

8. MOUSE EXPERIMENTS

(a) Inoculation of mice

CFLP mice (ICI strain, outbred) were obtained from Interfauna U K Ltd , Huntingdon, Cambs CD-1 random-bred mice were obtained from Charles River U K Ltd , Margate, Kent Balb/c mice were obtained from B&K Ltd , Hull, N Humberside Mice were male, and were used at 5 weeks of age Groups of 7 to 10 mice were inoculated intranasally under light ether anaesthesia with 20µl of DI SFV in 10 LD_{50} (1500 p f u) SFV, after the method described by Dimmock and Kennedy (1978) A single dose was delivered and mice were examined daily until the onset of disease (usually after 4 to 6 days), after which they were examined at least twice daily for signs of sickness, paralysis and death until the course of the disease was over (usually 10 days after infection) Mice were challenged with 100 LD_{50} SFV approximately 20 days after the first inoculation to determine their

immune status. All inoculations were performed by Professor N.J. Dimmock (Department of Biological Sciences, University of Warwick).

(b) Dissection of mice

Mice were killed with ether. Brains were dissected and snap-frozen with dry-ice and transferred to -70°C for storage.

CHAPTER 3

DETECTION OF DEFECTIVE RNAs OF SEMLIKI FOREST VIRUS USING THE POLYMERASE CHAIN REACTION

INTRODUCTION

This chapter describes the detection of DI SFV RNA by reverse transcription and amplification using the polymerase chain reaction (RT-PCR). The optimisation of these techniques is described and their sensitivity and specificity appraised. The relative merits of RT-PCR and northern analysis for the detection of DI SFV genomes, and their application to the detection of DI SFV genomes in brain tissue from mice is described.

The primary aim of this project was to isolate and characterise mouse-protecting DI SFV sequences. DI SFV populations are known to be heterogeneous (Kääriäinen *et al.*, 1981), and some consist of both protecting and non-protecting species (Barrett *et al.*, 1984a). It was suspected that protection of mice by co-administration of SFV and DI SFV involved preferential amplification of the protecting DI SFV species. From this premise, there was a greater probability that DI genomes isolated *in vivo* had protecting activity than those isolated *in vitro*. Hence it was envisaged that the *in vivo* system could be used as a filtering mechanism for protecting sequences and the initial approach was to attempt to isolate DI SFV genomes from protected mice.

Initially it was necessary to choose a method that was applicable to the detection and isolation of DI SFV. Previous attempts to detect DI SFV *in vivo* have been largely unsuccessful. The one exception involved an amplification step in tissue culture, but this method was too impractical for routine use (Dimmock and Kennedy, 1978). Barrett and Dimmock (1981) described an RNA synthesis inhibition assay (RSIA), which detected ~$10^{3.75}$ DI particles per ml. However,

application of this method to the detection of DI SFV *in vivo* was unsuccessful. It was suspected that in protected mice, DI SFV was present in low concentration and this assay was not sufficiently sensitive (Barrett *et al.*, 1984c). In 1985, Saiki *et al.* reported the development of the polymerase chain reaction (PCR). Since then, PCR has been used extensively for diagnostic purposes, having high specificity and sensitivity, and was therefore an appropriate technique, when coupled with reverse transcription, to use for the detection of DI SFV RNAs *in vivo*.

The technique of RT-PCR was optimised before applying it to the detection of DI SFV genomes. This optimisation was dependent in part on having an efficient method for extracting RNA. The RNA extraction methods that were used and their relative merits are discussed in this chapter because of their importance in establishing a reliable method for detecting DI SFV genomes. To optimise RT-PCR it was necessary to establish a method for determining the sensitivity of the technique and to permit subsequent modifications to be appraised. Primers were designed that were specific for internal sequences of SFV RNA and the sensitivity of the technique assessed from the lowest concentration of SFV RNA that could be detected using these primers. It was therefore possible to measure sensitivity in terms of the infectivity titre of the virus.

To extend the technique of RT-PCR to the detection of DI SFV genomes it was necessary to design primers that were specific for these genomes. In addition, to enable subsequent cloning and characterisation of DI SFV genomes, it was preferable to use primers that annealed to the termini so that the entire genome could be amplified. As stated in the General Introduction, previously sequenced genomes of DI SFV possessed termini derived from those of the standard RNA

These termini were conserved in all the DI SFV genomes studied (Lehtovaara *et al.*, 1981, 1982). It was reasonable, therefore, to expect that complete DI SFV genomes would be obtained by RT-PCR using primers specific for the termini of SFV RNA. Furthermore, because the genome of SFV is 11.4 kb, it was unlikely to be amplified under the conditions used for RT-PCR, or in preference to DI genomes.

RESULTS

1. Methods used to investigate DI SFV genomes

(a) RNA extraction methods

In preliminary experiments, RNA was extracted from large volumes (typically 15 ml) of tissue culture fluid. This involved high-speed centrifugation and treatment of pelleted virus with SDS and a proteinase to degrade coat proteins, which were removed by phenol extraction. While this method provided RNA of adequate purity for RT-PCR and sufficient quantities for northern analysis, it was impractical for extracting RNA from large numbers of samples. The method that was used subsequently involved treatment of tissue culture fluid with SDS and urea before extracting RNA with phenol. This method was applicable to small volumes (typically 75μl) of tissue culture fluid, and permitted large numbers of samples to be handled simultaneously. Furthermore, this method was rapid by comparison to the one described above, hence potential exposure to RNAses was reduced.

For analysis of viral RNAs in tissue culture cells or brain homogenates, total cellular RNA was used. This analysis was therefore made against a background of non-viral RNA. It was likely that non-viral RNA represented the majority, but may have been beneficial to the process of extraction, serving both to reduce degradation of specific RNA species and to increase the percentage of the viral RNAs that were successfully purified. However, as will be discussed later, it was likely that these high levels of background RNA reduced the sensitivity and specificity of the detection of DI SFV genomes by RT-PCR.

(b) Northern blotting

To detect SFV-derived RNAs by northern blotting, a virus-specific hybridisation probe was used. In the first experiments, an end-labelled DNA primer was used as the probe (*3'SFV*), which was complementary the 3' terminus of SFV RNA. However, it was not possible to detect SFV-derived RNAs with this primer, suggesting that the labelling was inadequate. In later experiments, a probe was used that was made by nick-translation of a DNA clone of DI SFV (see Chapter 4). While this probe was sufficiently sensitive for the detection of SFV RNAs, the profiles obtained by northern analysis of DI SFV RNA were usually poorly defined. This was either a consequence of RNA degradation, or a reflection of the heterogeneity of the DI RNA. Northern analysis was therefore not used as a standard technique for analysing DI SFV preparations, but was used subsequently in the study of homogeneous preparations of DI SFV (see Chapter 7).

(c) Optimisation of the polymerase chain reaction

In preliminary experiments, reverse transcription and PCR were performed as independent stages, but more reproducible results were obtained by combining these two stages in the same reaction mixture. PCR primers were designed that were specific for internal sequences of SFV RNA: these were designated *SFV-5i* (homologous to nucleotides 1780 to 1798 of SFV RNA) and *SFV-3i* (complementary to nucleotides 2512 to 2495 of SFV RNA). SFV RNA was prepared from a tissue culture preparation of SFV, and analysed by RT-PCR using these primers. A single product of 0.7 kbp was obtained, which matched the size of

the region of SFV RNA expected to be amplified using these primers (for an example, see Figure 3.1). Because this analysis was reproducible, the technique of RT-PCR was optimised using these primers. A number of experiments were performed in which different parameters of the RT-PCR protocol were varied. These included the incubation times and temperatures, and the concentration of enzymes, primers and magnesium ions. Optimal values for these parameters were estimated by visual examination of banded DNA on an agarose gel.

These first optimisation experiments established a method that was applicable to the detection of DI SFV genomes. Numerous modifications to the technique were made throughout the project to improve sensitivity and specificity (discussed below).

(d) Sensitivity and specificity of RT-PCR

The first experiment to determine the sensitivity of RT-PCR is shown in Figure 3.1. RNA was extracted from a tissue culture preparation of SFV, serially diluted, reverse transcribed and subjected to 30 cycles of PCR. From the infectivity titre of the virus preparation, and taking the highest dilution at which there was a visible product on an agarose gel as the end-point, it was determined that the sensitivity of RT-PCR was equivalent to 4×10^4 p f u. In a later experiment, the virus preparation itself was serially diluted and RNA extracted from the dilutions. Following ethanol precipitation, the RNA was resuspended directly in RT-PCR reaction mixture to minimise the potential exposure to RNAses. In addition, an RNAse inhibitor was included (see Methods). The sensitivity of RT-PCR using this method was equivalent to 1×10^1 p f u.

Figure 3.1 Determination of the sensitivity of RT-PCR

RNA was extracted from a tissue culture preparation of SFV and serially diluted in two-fold steps ranging from the equivalent of 1 x 10^5 p.f.u. (lane 1) to 2 x 10^4 p.f.u. (lane 10). The RNA was reverse transcribed and subjected to 30 cycles of PCR, using the primers *SFV-5i* and *SFV-3i*. PCR products were analysed on a 1% agarose gel. The highest dilution of RNA at which the 0.7 kbp product was visible by ethidium bromide staining was equivalent to 4 x 10^4 p.f.u. (lane 9).

A number of attempts were made to improve the sensitivity of RT-PCR. Increasing the number of PCR cycles had little effect, suggesting that the efficiency of the reaction was negligible after 30 cycles. Experiments with re-amplification were unsuccessful. In these, a ten-fold dilution series of PCR products was made, ranging from 1/10 to $1/10^4$. PCR amplification of these dilutions yielded heterogeneous products, and small DNA products that were undetected after the first amplification were preferentially amplified in the second (data not shown).

Specificity was likely to be dependent on a number of factors, including the design and concentration of the PCR primers, and the temperature used to anneal the primers. The melting temperature of annealed primers was estimated from their base-content, using the formula T_m (°C) = 4(G + C) + 2(A + T) (derived from Marmur and Doty, 1962). In general, primers were annealed 2 to 5°C below the T_m. Because the reverse transcription stage was performed at 42°C and the estimated melting temperature for most primers was approximately 20°C higher than this, non-specific hybridisation was likely to occur during this stage. This problem arose frequently when RT-PCR was used to amplify from total cellular RNA samples, which comprised a high proportion of non-specific RNAs. A number of attempts were made to improve the specificity of primer annealing during reverse transcription. These included reducing the concentration of primers during this stage, adding the homologous primer after reverse transcription, reducing the time of incubation at 42°C and reducing the concentration of reverse transcriptase. However, these modifications were not beneficial because specificity was improved only marginally with a concomitant reduction in sensitivity.

2. Detection of DI SFV genomes by RT-PCR

(a) Detection of DI SFV genomes in tissue culture fluid

For the detection of DI SFV genomes, primers were designed that were specific for the termini of SFV RNA. These were designated *5'SFV* and *3'SFV*. In preliminary experiments, a preparation of DI SFV known to interfere *in vitro* with standard virus propagation was used as a parent stock for preparing sufficient quantities of tissue culture fluid for analysis by RT-PCR. One such preparation, designated p4-2/4, was produced by four serial passages of this stock in BHK-21 cells. Figure 3.2a shows DNA derived from amplification of p4-2/4 RNA by RT-PCR. A single product of 1.5 kbp was obtained. Comparison of this product with those derived from non-infected or SFV samples showed that it was unique to the DI SFV sample (data not shown).

Figure 3.2b shows the PCR products derived from tissue culture fluid that was produced by serial undiluted passage of SFV in BHK-21 cells. The SFV preparation in this experiment was produced by transfection of RNA transcripts of the full-length SFV clone pSP6-SFV4 into BHK-21 cells (Liljeström *et al.*, 1991). The 0.8 kbp product derived from passage 9 was not present in those derived from passages 1 and 2. In addition, only passage 9 was shown to have a significant interference titre in tissue culture (see Chapter 7). Although at this stage there was no proof that these PCR products comprised complete DI SFV genomes, these results suggested that DI SFV genomes were detected by RT-PCR using the primers *5'SFV* and *3'SFV*. It was reasonable, therefore, to extend the investigation to the detection of DI SFV *in vivo*.

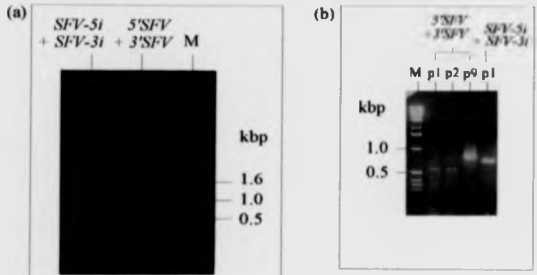


Figure 3.2 Detection of DI SFV genomes in tissue culture fluid

RNA was extracted from a tissue culture preparations of DI SFV reverse transcribed and subjected to 30 cycles of PCR using primers to detect SFV RNA (*SFV-5i* + *SFV-3i*) or DI SFV RNA (*5'SFV* + *3'SFV*). In **(a)** the preparation p4-2/4 was used (see text for details). A 0.7 kbp product was generated with the primers *SFV-5i* + *SFV-3i*, and one of 1.5 kbp with the primers *5'SFV* + *3'SFV*. In **(b)** preparations were made by serial passage in BHK-21 of SFV derived from pSP6-SFV4 (Liljeström *et al.*, 1991). After RT-PCR using the primers *5'SFV* + *3'SFV*, DNA species of 0.6 kbp were obtained from passages (p) 1 and 2, and a relatively abundant species of 0.8 kbp from passage 9. A 0.7 kbp product was obtained from passage 1 using the primers *SFV-5i* + *SFV-3i*.

(b) Detection of DI SFV genomes *in vivo*

For these experiments, a mouse-protecting tissue culture preparation of DI SFV was used. This preparation, designated DI SFV-p7, was produced by 7 serial passages of SFV in BHK-21 cells. In a typical mouse-protection experiment, approximately 70% survival was observed after intranasal inoculation of DI SFV-p7 and 10 LD_{50} SFV into adult mice (see Chapter 8). In the experiment shown in Figure 3.3, mice were dissected 2 or 4 days after inoculation. RT-PCR was performed on total cellular RNA that had been extracted from brain tissue, and the primer pairs *SFV-5i + SFV-3i* were used for detecting virus, or *5'SFV + 5'SFV* for detecting DI virus. Virus was detected only in the samples derived from mice inoculated with SFV or with 100 LD_{50} SFV + DI. From all samples analysed with the primers *5'SFV + 3'SFV*, numerous low molecular weight PCR products were obtained, the agarose gel profiles of which did not vary significantly between samples. These were therefore suspected to have been generated as a consequence of the primers hybridising to mouse-derived RNAs, and DI genomes had not been detected. RT-PCR was repeated with a higher primer annealing temperature to increase specificity, and although the number of low molecular weight products was reduced, there was still no difference between the PCR products derived from the DI + SFV samples and the others.

In Figure 3.4, total brain RNA from the experiment described above was analysed by northern blotting. The RNA used was derived from mice 2 days after inoculation, and was probed with a nick-translated DI SFV clone (pSFVDI-7, see Chapter 4). The profile of the sample derived from mice inoculated with 100 LD_{50} SFV + DI differed from the control samples, with two indistinct bands of

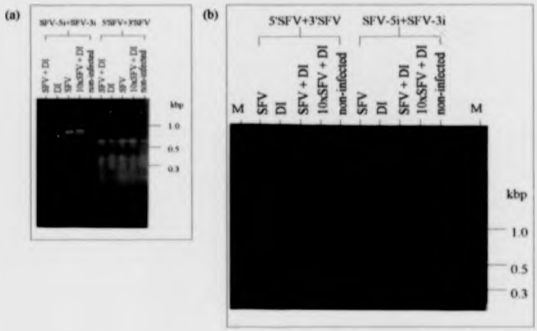


Figure 3.3 RT-PCR of total RNA derived from mouse brain tissue

Adult mice (male CFLP) were inoculated intranasally, and brains removed 2 or 4 days afterwards. RNA was extracted with guanidinium isothiocyanate and 5µg (approximately one twentieth) used in RT-PCR. 30 cycles of PCR were performed, using the primers *SFV-5i* + *SFV-3i* to detect virus, or *5'SFV* + *3'SFV* to detect DI virus. **(a)** and **(b)** show PCR products derived from brain RNA extracted 2 or 4 days after inoculation. A 0.7 kbp product was obtained from samples derived from mice inoculated with SFV alone (10 LD_{50}) or DI + 10xSFV (100 LD_{50}) using the virus-specific primers. Amplification with *5'SFV* + *3'SFV* yielded products of 0.3 to 0.6 kbp from all samples.

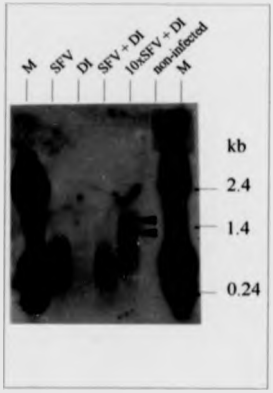


Figure 3.4 Northern blot analysis of total RNA derived from mouse brain tissue

Adult mice (male CFLP) were inoculated intranasally, and brains removed 2 days afterwards. RNA was extracted with guanidinium isothiocyanate and 10μg electrophoresed on a 1% agarose gel after denaturation with glyoxal. Following northern blotting, RNA was hybridised with a nick-translated DI SFV clone labelled with [α-^{32}P]-dATP (pSFVDI-7; see Chapter 4). This probe also contained sequences derived from the vector, which hybridised to some RNA species of the unlabelled size marker. Two indistinct bands corresponding to 1.0 and 1.5 kb (arrowed) were produced by hybridisation of the probe to RNA derived from mice inoculated with DI + 10xSFV (= 100 LD_{50})

approximately 1.0 and 1.4 kb. Because the northern blot was poorly defined, it was not possible to make any firm conclusions on the nature of this highlighted RNA.

(c) Detection of DI SFV genomes in mouse-protecting tissue culture preparations

Because it was not possible by the methods used to detect DI SFV genomes *in vivo*, attempts were made to detect DI genomes in the tissue culture preparations that were used as inocula for mouse-protection experiments. In preliminary experiments, the mouse-protecting preparation DI SFV-p7 was passaged once in CEF cells to generate sufficient quantities of tissue culture fluid for analysis by RT-PCR. Figure 3.5 shows a PCR product derived from this preparation (designated DI SFV-p8), which contains a predominant DNA species of 2.1 kbp. In a similar experiment, a different preparation of DI SFV was used (DI SFV-AC). This had been made previously by passaging a mouse-protecting DI SFV preparation in BHK-21 cells. A single product of 2.1 kbp was also obtained by RT-PCR amplification of RNA extracted from this preparation.

Once the method for extracting RNA from small volumes of tissue culture fluid had been established (see section 1a), it was possible to analyse the mouse-protecting preparation DI SFV-p7 itself. Figure 3.6 shows the PCR product derived from this preparation and a comparison with the one derived from DI SFV-p8. The predominant DNA product derived from DI SFV-p7 was one of 1.2 kbp, which contrasts with the 2.1 kbp product from DI SFV-p8. In addition, while DI SFV-p7 protected adult mice against a lethal dose of SFV, DI SFV-p8 did not.

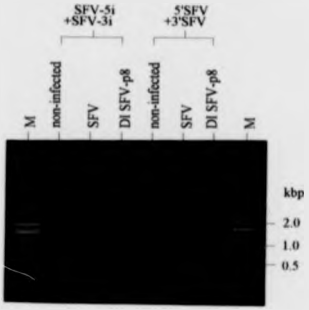


Figure 3.5 Detection of DI SFV RNA in the tissue culture preparation DI SFV-p8

CEF cells were incubated with medium alone, SFV (ts1 strain) or DI SFV-p7 + SFV. Tissue culture fluid was harvested after 24 hours and RNA extracted, reverse transcribed and subjected to 30 cycles of amplification by PCR. The primers *SFV-5i* + *SFV-3i* were used to detect virus, or *5'SFV* + *3'SFV* to detect DI virus. A 0.7 kbp product was obtained from the SFV-alone and DI SFV-p8 samples using the primers *SFV-5i* + *SFV-3i*. RT-PCR using *5'SFV* + *3'SFV* yielded a 2.1 kbp product from DI SFV-p8.

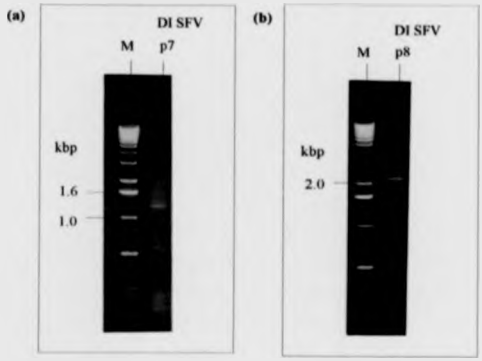


Figure 3.6 Comparison of PCR products derived from DI SFV-p7 and DI SFV-p8

DI SFV-p8 was produced from the mouse-protecting tissue culture preparation DI SFV-p7 by passaging once in BHK-21 cells. RNA from both these preparations was amplified by RT-PCR using the primers *5'NV* + *3'NV*. A predominant product of 1.2 kbp was obtained from DI SFV-p7 **(a)**, and one of 2.1 kbp from DI SFV-p8 **(b)**

DISCUSSION

Of the two methods that were used to extract RNA from tissue culture fluid, the one used for small volumes was suspected to be more efficient because a lower concentration of SFV RNA was detected by RT-PCR. It was suspected that non-viral RNAs represented a large proportion of the total, and reduced the efficiency of RT-PCR. In addition, the total amount of RNA that was used in a reaction was thought an important determinant of efficiency. For example, in Figure 3.1 the intensity of the bands comprising the PCR products was reduced in samples derived from the highest amounts of RNA. In later experiments, it was shown that above RNA concentrations of 20μg per reaction it was not possible to detect SFV RNA, suggesting that RT-PCR was totally inhibited. Inhibition of PCR by non-specific nucleic acid has been demonstrated before. For example, Abbott *et al* (1988) used PCR to detect HTLV-I DNA and found that the limits of detection of the technique were reduced from 1 ng to 10 ng with the addition of 1μg non-specific DNA.

The sensitivity of RT-PCR was determined primarily to permit optimisation of the technique. Although sensitivity was determined for the detection of viral RNA, the value obtained was not applicable to the detection of DI SFV genomes, for various reasons. First, the primers that were used were different, and it was possible they had a different affinity for the template. In addition, because DI preparations are heterogeneous, it was likely that the primers used to detect DI virus annealed to a variety of templates, including the virion RNA. While this was unlikely to deplete the pool of primers because they were at a sufficiently high concentration, the overall efficiency of RT-PCR may have been reduced. Finally, the size of the

products were in general larger than the product obtained with the virus-specific primers. Because smaller templates are amplified preferentially, the sensitivity determined using the virus-specific primers was likely to be greater than that using the primers for detecting DI virus.

DI genomes were not detected *in vivo*, but it was possible that RT-PCR was not sufficiently sensitive. The sensitivity of RT-PCR was determined to be equivalent to 10^3 p.f.u. virus, and applied to the detection of DI virus, it was unlikely to be more sensitive than methods used previously, for the reasons described above. Because DI virus is dependent on standard virus for propagation, it was likely that *in vivo*, levels of DI SFV were determined by the levels of infectivity. During mouse-protection, it was suspected that the DI virus was present only in amounts necessary to prevent the spread of residual infectivity. However, by inoculating with a high dose of SFV to counteract the protecting effect, it was hoped that the DI virus would be propagated to levels that would be detected. While northern blot analysis of these samples suggested DI virus was present in mouse brain, detection with RT-PCR was unsuccessful. It was suspected that one of the problems of this analysis was the high proportion of non-viral RNA in the samples that were analysed, which was reflected by the many different PCR products that were obtained by non-specific hybridisation of the PCR primers. The proportion of non-viral RNAs could have been reduced by purifying the polyadenylated ones, which were likely to include putative DI RNAs. Alternatively, the sensitivity and specificity of RT-PCR could have been improved by using a nested set of PCR primers. However, at the time the nucleotide sequence of the protecting DI genomes, which was required for designing appropriate primers, was not known.

In contrast, it was possible to detect defective RNAs in tissue culture fluid, presumably because the ratio of DI RNA to non-viral RNA was higher. It was not known at this stage if the RNAs that were amplified by RT-PCR comprised complete genomes, or if they contained the sequences responsible for interference with standard virus propagation. However, the agarose gel profiles of PCR products derived from different tissue culture preparations concurred with differences in biological activity. This suggested that these PCR products were representative of the components of the preparations responsible for such activity. Furthermore, the loss of mouse-protecting activity on passaging DI SFV-p7 coincided with a reduction in levels of the 1.2 kbp PCR product derived from the tissue culture fluid. This suggested that this DNA species contained nucleotide sequences derived from a mouse-protecting DI virus. This PCR product was approximately half the size of previously cloned DI SFV genomes (Lehtovaara *et al.*, 1981; 1982). It was possible that it comprised a repeated unit of a larger genome, but at this stage of the project there was no evidence for this.

In general, just one product was detected after RT-PCR amplification of tissue culture fluid. Because DI SFV populations are heterogeneous, it was likely that this was a consequence of preferential amplification of small or abundant RNAs. Most DI SFV genomes studied previously comprise less than 3 kb. In later experiments with RT-PCR it was possible to amplify sequences of 3 kb, suggesting that the method was applicable to the detection of most DI SFV genomes. Indeed, defined PCR products were generated from every DI SFV preparation that was examined. However, for RNA preparations comprising differently sized DI genomes (for example, those from DI SFV-p7) it was likely that the relative amounts of PCR products derived from these did not reflect the amounts of the respective RNA

species. It was possible that the use of primers specific for the termini of SFV RNA excluded certain RNA species from amplification by RT-PCR. However, evidence from previously analysed DI SFV genomes suggested that all retained the termini of SFV RNA (Lehtovaara *et al.*, 1981, 1982). As a consequence of these limitations of RT-PCR, it was unlikely that the PCR products were an accurate representation of the DI SFV species in the RNA preparations from which they were derived. It would have been desirable to develop the technique of northern blotting for analysis of all the SFV-derived RNAs in a given preparation, but for the purposes of the project this was unnecessary. It was suspected that mouse-protecting DI SFV genomes would comprise a substantial proportion of the total SFV-derived RNA from tissue culture fluid and therefore would be amplified preferentially by RT-PCR. A further advantage of using RT-PCR was that sufficient quantities of DNA were produced to enable cloning and subsequent analysis of putative DI SFV genomes.

CHAPTER 4

MOLECULAR CLONING OF DEFECTIVE GENOMES OF SEMLIKI FOREST VIRUS

INTRODUCTION

This chapter describes the molecular cloning of PCR products derived from mouse-protecting DI SFV tissue culture preparations. Modifications of two of these clones, pSFVDI-6 and pSFVDI-19, are also discussed. In addition, the construction of various sub-clones of pSFVDI-6 and pSFVDI-19 and the construction of an artificial DI genome by the internal deletion of the full-length infectious clone pSP6-SFV4 (Liljeström *et al.*, 1991) are described. It should be noted that this chapter is included to show how the project progressed and therefore includes results that were obtained without the benefit of hindsight.

The purpose of constructing clones was to permit characterisation of DI SFV genomes by sequencing, and to enable homogeneous defective virus populations to be produced, so that their biological activities could be defined on the basis of nucleic acid content. To date, only a few DI SFV clones have been made (discussed in Chapter 5), these are incomplete, lacking the 5' termini of the defective genomes (Lehtovaara *et al.*, 1981, 1982). Because RT-PCR was used in this project to amplify DI SFV genomes using primers to the termini of SFV RNA, the amplification products retained the termini of the defective genome. Assuming that there were no other sequences either side of the DI RNA from which the PCR products were derived, it was reasonable to expect that clones of these PCR products would comprise complete DI SFV genomes and hence that RNA transcripts from the clones could be propagated as DI virus.

Initially PCR products were cloned into a transcription vector and RNA transcribed from these clones had additional sequences derived from this vector at

both termini. Similar work with DI Sindbis virus by Levis *et al.* (1986) showed that biologically active RNA transcripts were produced if the promoter for RNA polymerase was positioned directly upstream of the insert, and the restriction enzyme site that permitted run-off transcription directly downstream. No work was presented on the consequence of having additional non-viral sequences attached to the RNA transcripts, but Liljeström *et al.* (1991) found that the presence of non-viral sequences upstream of the full-length SFV clone was deleterious to infectivity. For example, the clone pSP6-SFV3 had two extra C-residues between the insert and the transcription start site, the removal of which increased infectivity 1000-fold, to levels comparable to that of the virion RNA. whether these findings have any bearing on the propagation as virions of DI SFV genomes is not known. However, it was found that RNA transcripts derived from the first clones that were made were not propagated as virions. It was not known whether this non-propagation was due to the presence of additional sequences at both termini or because an efficient method for transfecting RNA transcripts into tissue culture cells had not been established at this stage of the project. As a precaution, these clones were modified so that RNA transcripts produced from them contained only SFV-derived sequences.

The method for obtaining clones with correctly aligned transcription features was based on that used by Makino *et al.* (1990). They produced clones of defective mouse hepatitis virus from PCR products generated using a 5' primer that incorporated the promoter for T7 RNA polymerase and a 3' primer containing a unique restriction enzyme site. Hence the transcription start site of the clone obtained, and the location of the 3' cleavage site that permitted run-off transcription, was determined in the design of the PCR primers. This chapter

describes the use of such primers for the modification of existing clones and describes the cloning of PCR products obtained from RNA using these primers (see Chapter 4)

The second part of this chapter describes the construction of various sub-clones of two of the full-length DI SFV clones that were produced (pSFVDI-6 and pSFVDI-19). These sub-clones were made primarily for sequencing purposes (see Chapter 5). Two internally deleted sub-clones of pSFVDI-6 (pSFVDI-1A2 and pSFVDI-M2) were used subsequently to investigate regions of the DI genomes that were of importance in interference and propagation (Chapter 7).

Finally, attempts were made to construct an artificial DI SFV genome from the full-length SFV clone, pSP6-SFV4 (Liljeström *et al.*, 1991). The only previous report of an artificially constructed DI genome was for poliovirus (Kaplan and Racaniello, 1988; Hagino-Yamagishi and Nomoto, 1989). An infectious DNA clone of poliovirus was cleaved with an appropriate restriction enzyme such that the region encoding capsid proteins was deleted and the non-structural protein coding region remained intact. RNA transcribed from this clone was capable of autonomous replication, but required helper poliovirus for encapsidation. Insertion of a 4-base sequence into the clone caused a frame-shift that prevented the RNA transcripts from being propagated. However, evidence from previously cloned DI SFV and Sindbis genomes suggested that for these viruses it was not important to retain protein-coding regions (SFV: Lehtovaara *et al.* 1981, 1982; Sindbis: Levis *et al.*, 1986). For cloned DI SFV genomes, the regions derived from the termini of the standard virus genome were found to be conserved, as were the regions derived from the nsP2 gene of SFV (Jalanko and Söderland, 1985). Because it was not

certain which of these regions were essential for their propagation as virions, the clone described in this chapter (pSFVDI-SB1) was constructed so that only the termini of the SFV-derived sequences of pSP6-SFV4 were retained. The propagation in tissue culture of RNA transcripts produced from this clone is discussed in Chapter 7.

RESULTS

1. Cloning of DI SFV-derived PCR products.

(a) Cloning of PCR products

PCR products derived from tissue culture preparations of DI SFV (see Chapter 3) were purified by phenol extraction and then modified so that they could be cloned. Firstly, because the PCR primers used for the amplification of DI SFV genomes lacked the 5' terminal phosphate group the products were treated with T4 polynucleotide kinase. In addition, it was assumed that the products of PCR amplification had ragged ends as a consequence of the *Taq* polymerase incorporating nucleotides less efficiently at the termini of the template DNA. Although the final step of 5 minutes at 72°C in the protocol used for PCR probably helped the terminal incorporation of nucleotides, products were treated with the large fragment of *E.coli* DNA polymerase I to ensure that they were blunt-ended.

The initial choice of vector for cloning was pBluescribe II KS$^+$, which has a polylinker incorporated into the gene for β-galactosidase, and an ampicillin resistance gene for selecting transformed bacteria. Colonies harbouring recombinants were distinguished from those with just vector DNA by their absence of colour when grown on agar containing IPTG and X-gal (see Methods). This vector contains the promoters for T3 and T7 RNA polymerases, enabling transcripts of cloned inserts to be produced. For reasons outlined in the Introduction, later experiments with PCR used a 5' primer containing the promoter for T3 RNA polymerase (*5'SFV-T3*), and a 3' primer containing the restriction

enzyme site NcoI (*SV-Nco*). The vector chosen for the cloning of these PCR products and for the majority of the work described here was pUC13, which has the same selection features as pBluescribe II KS$^+$, and does not possess promoters for RNA polymerases. Both the vectors described above possess a site for the restriction enzyme SmaI within the polylinker region. This enzyme cleaves the site at the axis of dyad symmetry, producing blunt ends. Hence PCR products that had been blunt-ended were inserted into vector linearised with SmaI.

(b) Construction of pSFVDI clones 1-8

The previous chapter showed that similarly sized (2.1 kbp) products were obtained following RT-PCR amplification of RNA extracted from two different tissue culture preparations of DI SFV (DI SFV-p8 and DI SFV-AC). Both these preparations had biological activity *in vitro* and were derived from populations that had mouse-protecting activity, although they were non-protecting themselves (see Chapter 8). At this stage of the project it seemed worthwhile to clone these PCR products because no full-length clones of DI SFV genomes had been produced before, and because it was suspected that the tissue culture preparation from which they were derived was heterogeneous and consisted of both protecting and non-protecting DI virus. Initially clones were obtained of PCR products derived from both the above DI SFV preparations by blunt-end ligation into the SmaI site of pBluescribe II KS$^+$ and transformation into *E. coli* (TG2) cells. In the first cloning experiment a recombinant plasmid was isolated containing a 2.1 kb insert, which was confirmed to be derived from PCR product by Southern blotting using a [γ-^{32}P]dATP-labelled PCR primer as probe (data not shown). This clone was designated pSFVDI-1.

Figure 4.1, taken from a cloning experiment that used PCR product derived from DI SFV-p8, shows a number of plasmid preparations from bacteria potentially harbouring recombinant DNA. The plasmids shown in lanes 1, 2, 6 and 9 all contained a 2.1 kbp insert which, like pSFVDI-1, was cut once asymmetrically by PvuII: these were designated pSFVDI-3 to pSFVDI-6. The other recombinant plasmids shown in this figure, containing inserts of between 0.2 and 1.2 kbp in size, were either artefacts of the methodology employed for RT-PCR and cloning or represented DI sequences smaller than the predominant PCR product described above. One such clone of 0.6 kbp in size was analysed by sequencing and found to consist only of the terminal sequences of SFV RNA, suggesting on comparison with published DI SFV genomes that it was unlikely to be a true DI genome (data not shown). These small clones were not investigated further. Similar cloning experiments not detailed here, using PCR products derived from DI SFV-AC, produced the clones that were designated pSFVDI-2, 7 and 8. These plasmids also had inserts of 2.1 kbp.

(c) Derivation of pSFVDI inserts

The first indication that the pSFVDI clones had inserts derived from a DI genome was through restriction analysis. Figure 4.2a shows examples of the restriction digests that were made. The AccI digest produced DNA fragments of 0.3 and 0.2 kbp, which were determined from double digests not shown here to be from juxtaposed regions in the clones. This suggested by comparison to a restriction map of the SFV genome (Figure 4.2c) that the clones contained at least 0.5 kbp derived from the 5' terminus of the SFV genome. In addition, three of the sites for AccI, PstI and PvuII were calculated to lie within approximately 100 bp of each

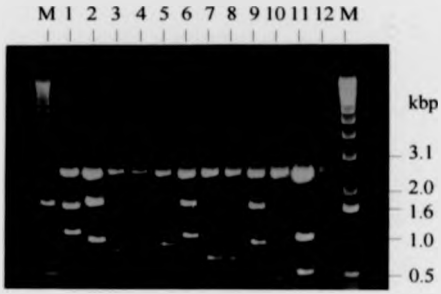
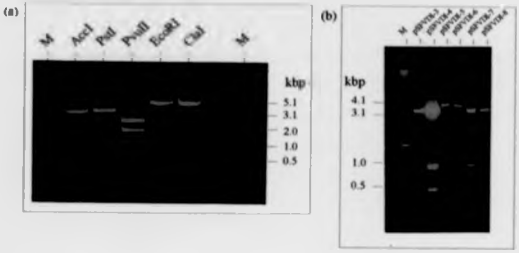


Figure 4.1 Agarose gel profile of PvuII-digested plasmid preparations from putative DI SFV clones

PCR product derived from DI SFV-p8 (Chapter 3) was cloned into the SmaI site of pBluescribe II KS^+ (3.0 kbp). Plasmid DNA was prepared from bacterial clones and cleaved with PvuII, which cuts pBluescribe II KS^+ either side of the polylinker to yield fragments of 2.7 kbp and 0.3 kbp. Inserted DNA was thus incorporated on the 0.3 kbp fragment. Twelve plasmid preparations are shown above. In all lanes, the 2.7 kbp band corresponds to the size of the vector-derived fragment, and the smaller bands to inserted DNA that includes 0.3 kbp from the vector. The inserts were calculated to range in size from 0.2 to 2.1 kbp. Four of the plasmids (lanes 1, 2, 6, 9) had inserted DNA of 2.1 kbp, corresponding to the size of the predominant PCR product from DI SFV-p8.



(c) Semliki forest virus

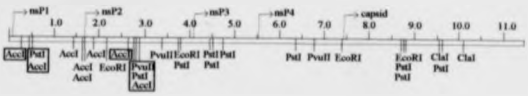


Figure 4.2 Restriction analysis of DI SFV clones

(a) shows examples of restriction digests of pSFVDI-6 (5.1 kbp) that were made to establish the origin of the inserted DNA. (b) shows six plasmid preparations digested with PstI: this enzyme cleaves the vector (pBluescribe II KS$^+$, 3.0 kbp) upstream of the SmaI site, and cleaves the inserted DNA twice. This figure shows that the inserted DNA is the same size in all six clones, and for pSFVDI-3, 7 and 8 is in one orientation within the vector, and for pSFVDI 4, 5 and 6 in the other. (c) shows a diagram of SFV cDNA, with relevant restriction sites indicated. Sites suspected to have been retained by pSFVDI-6 are boxed.

other. This close arrangement of restriction sites also occurs in the nsP2 gene of SFV, suggesting that the clones may contain part of this gene, and therefore that the cloned inserts were unlikely to be artefacts of the methodology used to produce them. Figure 4.2b shows an analysis of six of the clones digested with the enzyme PstI. This and other restriction analyses not shown here suggested that the clones pSFVDI -1 to 8 contained the same insert, either in the 5'-3' or 3'-5' orientation.

(d) Modification of pSFVDI-6

pSFVDI-2, 3 and 5 were found by sequence analysis to have lost nucleotides from the termini, probably in the process of cloning. The other clones had intact termini, and from these pSFVDI-6 was chosen for modification. Rather than returning to the original population of DI virus from which pSFVDI-6 was derived, it was thought more practical to amplify DNA from the clone itself. The primers *5'SFV-T3* and *3'SFV-Nco* were used in the amplification, and a 2.1 kbp PCR product was obtained (see Chapter 3), which was blunt-end ligated into the SmaI site of pUC13. It was necessary to remove the original vector before transforming bacteria by digesting with BamHI and EcoRI, which do not cut within the insert. Successful re-cloning of the insert in pSFVDI-6 was confirmed initially by restriction analysis (data not shown). To ensure the termini of the insert were intact, the plasmid was cut with NcoI and transcribed with T3 RNA polymerase (Chapter 6). Sequencing provided the final confirmation that the molecule had been successfully re-cloned.

(e) Construction of pSFVDI-19

RT-PCR amplification from the preparation DI SFV-p7 using the primers *5'SFV-T3* and *3'SFV-Nco* yielded a product of 1.2 kbp, in addition to one of 2.1 kbp (see Chapter 3). It was assumed that the 2.1 kbp product was the same as that observed on amplification from DI SFV-p8, and hence the same as the insert in pSFVDI-6. In addition, it was observed that while the passaging of DI SFV-p7 once in BHK-21 resulted in the loss of the 1.2 kbp product and the concomitant loss of mouse-protecting activity (see Chapter 8), the 2.1 kbp product was retained. It was reasonable to suspect, therefore, that the 1.2 kbp product comprised all or part of the protecting activity of DI SFV-p7 and that the 2.1 kbp product and therefore the insert in pSFVDI-6 had little or no such activity. This conclusion assumed that mouse-protecting sequences of DI SFV-p7 were indeed amplified by RT-PCR. The PCR product was transcribed prior to cloning (see Chapter 6) and the RNA transcripts transfected into SFV-infected BHK-21 cells to produce DI virions (see Chapter 7). The observed protecting activity of this DI SFV preparation in mice on coinoculation with a lethal dose of SFV (see Chapter 8) confirmed the above conclusion.

Figure 4.3 shows restriction digests of three preparations of recombinant plasmids from a cloning experiment that used the PCR product obtained from DI SFV-p7. The vector used here was pUC13 (2.7 kbp), and initial analysis of these recombinants by digestion with PvuII showed that two of these plasmids contained a 1.2 kbp insert. One of these two plasmids could be digested with NcoI, indicating that the 3' terminus of the insert was intact in this case: this was analysed further by transcription and sequencing. Unfortunately, transcription from this plasmid was

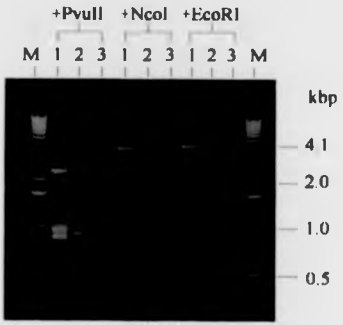


Figure 4.3 Restriction digests of plasmid preparations from putative DI SFV clones

Three potential DI SFV clones (1, 2 and 3) are examined here. These were produced by inserting the PCR product derived from DI SFV-p7 (Chapter 3) into the SmaI site of pUC13 (2.7 kbp). Digestion with PvuII, which cleaves either side of the pUC13 polylinker produced vector-derived fragments of 2.4 kbp, and fragments that totalled 1.8 kbp (1) and 1.5 kbp (2, 3) in size, these included 0.3 kbp derived from pUC13. Plasmids 2 and 3 therefore contained inserted DNA of 1.2 kbp in size, corresponding to the PCR product of interest. Only plasmids 1 and 3 were linearised with NcoI. EcoRI, which cleaves pUC13 once within the polylinker, did not cleave within the inserted DNA.

unsuccessful, suggesting that the 5' terminus containing the T3 RNA polymerase promoter was not intact. It was therefore necessary to re-clone. Because of limited starting material, more PCR product was made from the above plasmid, using the same primers as before. A clone was generated from this PCR product that had correct termini, as determined by transcription and sequencing experiments, and this was designated pSFVDI-19.

(f) Derivation of pSFVDI-19

Before it was sequenced, pSFVDI-19 was digested with a number of restriction enzymes, primarily to confirm the origin of the insert by comparison to digests of the PCR product from which it was derived, and in an attempt to elucidate the regions in the SFV genome from which this putative DI genome was derived. It seemed likely from these digests that the pSFVDI-19 insert had derived from the 1.2 kbp PCR product and further that it contained part of the nsP2 gene, for the same reasons described above for pSFVDI-6 (data not shown).

(g) Restriction mapping of pSFVDI-6 and pSFVDI-19

As stated above, restriction sites were determined initially through a series of enzyme digestions. Figure 4.4 shows diagrams of pSFVDI-6 and pSFVDI-19, with relevant restriction sites included. To avoid confusion, the precise locations of these sites are shown: these were determined by computer analysis after the clones had been fully sequenced. The inserts of both clones are in the 3'-5' orientation and have unique NcoI sites at the 3' termini enabling the plasmids to be linearised before transcription. Unique restriction sites within the inserts themselves include

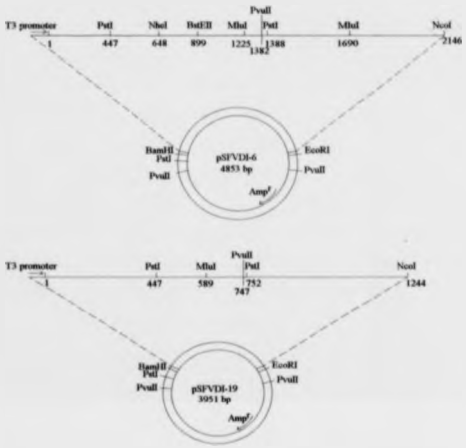


Figure 4.4 Diagrams of pSFVDI-6 and pSFVDI-19

DNA was inserted into the SmaI site of the pUC13 polylinker M13mp11. Both inserts are shown as linear segments, in the 5' to 3' orientation, and relevant restriction sites are indicated. The numbers shown refer to the nucleotides adjacent to, and on the 5' side of the points of cleavage. The segment shown below the dashed lines represents pUC13 DNA; the ampicillin resistance gene (Amp^r) is shown. The restriction sites BamHI, PstI and EcoRI of pUC13 are located within the polylinker.

those for NheI and BstEII in pSFVDI-6 and MluI in pSFVDI-19. Unique sites for BamHI and EcoRI are contained within the pUC13 polylinker.

2. Creation of sub-clones of pSFVDI-6 and pSFVDI-19

(a) Sub-cloning from pSFVDI-6

Sub-clones were produced from pSFVDI-6 which permitted the sequencing of the majority of this clone and hence provided the information needed for primers to be designed that could be used to determine the remainder of the sequence (see Chapter 5). The restriction enzyme PstI was found to generate the most conveniently sized fragments for this sub-cloning (Figure 4.5a). At this stage of the project, the insert of pSFVDI-6 had not been modified (see above) and was in a pBluescribe II KS$^+$ vector in the 5'-3' orientation. The PstI site in the pBluescribe II KS$^+$ polylinker was upstream of the 5' terminus of the unmodified insert, enabling the 5' terminal 0.5 kbp fragment and the internal 0.9 kbp fragment to be isolated from the clone. The methodology was therefore to digest pSFVDI-6 with PstI, isolate the 0.5 and 0.9 fragments by separation on an agarose gel, purify with Geneclean (see Methods), and ligate these into PstI-digested pBluescribe II KS$^+$. This produced the clones designated pSFVDI-6P1 and pSFVDI-6P2. In addition, the digested pSFVDI-6 was religated to produce pSFVDI-6P3, which contained only the 3' terminal 0.8 kbp of the original clone. Figure 5.1 (Chapter 5) shows how these sub-clones were used for sequencing.

Two other sub-clones of pSFVDI-6 were made which were subsequently used to investigate the sequences required for propagation of DI SFV genomes. The first,

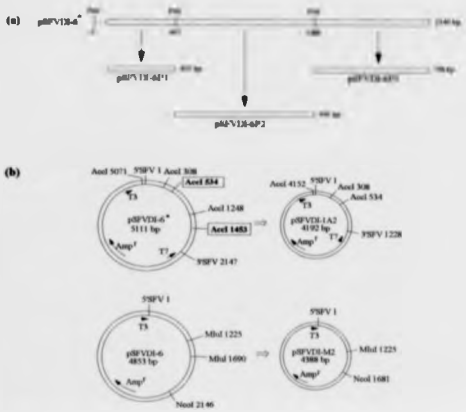


Figure 4.5 Diagrams of sub-clones of pSFVD1-6

(a) pSFVD1-6* was digested with PstI and the 451 and 941 bp fragments inserted into pBluescribe II KS$^+$ to give pSFVD1-6P1 and pSFVD1-6P2 respectively pSFVD1-6P3 was made by religation of PstI-digested pSFVD1-6*

(b) pSFVD1-1A2 was made by partially digesting pSFVD1-6* with AccI to delete the 919 nucleotide region between AccI 534 and AccI 1453 (boxed) pSFVD1-M2 was made by excision of the 465 bp MluI fragment of pSFVD1-6

* the original form of pSFVD1-6 was used here the vector was pBluescribe KS$^+$

pSFVDI-1A2, is shown diagrammatically in Figure 4.5b, and its profile on an agarose gel is shown in Figure 4.6a. To create this clone, pSFVDI-6 was partially digested by incubating for a brief period (2 minutes) with AccI and then religated. The AccI sites at 308 and 1248 are compatible, as are the ones at 534 and 1453, so the deleted forms of pSFVDI-6 that were produced lacked either the 940 region from 308 and 1248 or the 919 bp one between 534 and 1453. Digestion of pSFVDI-1A2 with PstI, which cleaves within the AccI (1248-1453) fragment of pSFVDI-6, showed that this fragment had been deleted (data not shown). Thus the 0.2 kbp band shown in lane 2 of Figure 4.6a comprised DNA derived from the AccI (308-534) fragment and the deletion in pSFVDI-1A2 comprised the region between AccI 534 and AccI 1453 of pSFVDI-6.

Figure 4.6a also shows other sub-clones that were made by the partial digestion of pSFVDI-6 with PstI, and religation. Two of these clones (shown in lanes 7 and 9) lacked the internal 0.9 kbp PstI fragment of pSFVDI-6, but because of their similarity to pSFVDI-1A2 they were not used in subsequent experiments.

pSFVDI-M2 was created to demonstrate that some function essential to the propagation of RNA transcripts of pSFVDI-6 was located between the two MluI sites (see Chapter 7). This clone was made by the excision of the 466 bp MluI fragment from pSFVDI-6 (Figure 4.5b). Figure 4.6b shows preparations of plasmid DNA from transformed bacteria, digested with either MluI or PvuII. Three of the four preparations shown were cut only once by MluI, indicating successful excision of the fragment. Further, digestion of these three with PvuII only gave two fragments, suggesting the site located between the two MluI sites was lost (see Figure 4.4) and that the deletion was successful.

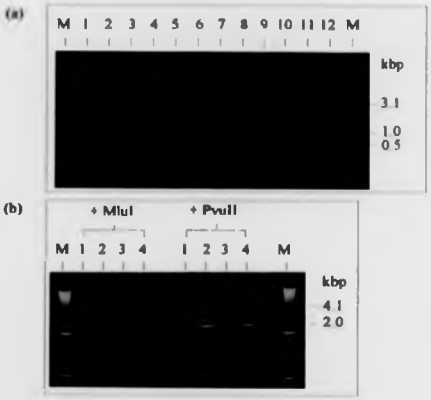


Figure 4.6 Agarose gel profiles of potential deletion mutants of pSFVDI-6.

(a) pSFVDI-6 was partially digested with AccI or PstI and transformed into bacteria. Plasmids from 12 recombinants were digested with AccI (1-6) or PstI (7-12). Of the AccI deletion mutants, all but clone 4 have lost either the 940 fragment between AccI 308 and AccI 1248, or the 919 fragment between AccI 534 and AccI 1453 (see Figure 4.5). Of the PstI deletion mutants, clones 7 and 9 have lost the internal 0.9 kbp PstI fragment of pSFVDI-6 (see Figure 4.4), clone 8 the 0.5 and 0.9 kbp fragments, and clones 10, 11 and 12 retained both these fragments.
(b) MluI-digested pSFVDI-6 was transformed into bacteria. Plasmids from clones 2, 3 and 4 were cleaved once by MluI to yield fragments of 4.4 kbp, and twice by PvuII to yield fragments of 2.1 and 2.3 kbp. The origin of clone 1 is not known.

(b) Sub-cloning from pSFVDI-19

Sub-clones from pSFVDI-19 were made solely for the purposes of sequencing. Three were made, by the same method as described above for pSFVDI-6 using PstI to generate suitable fragments for cloning (Figure 4.7). Unlike the sub-cloning from pSFVDI-6, however, the final modified version of pSFVDI-19 was used in the experiment. The plasmids that were generated were given the designations pSFVDI-19P1, 2 or 3. The first two were created by inserting the 5' terminal 0.5 kbp and the internal 0.3 kbp PstI fragments of pSFVDI-19 into pBluescribe II KS$^+$, while pSFVDI-19P3, which was generated through religating digested pSFVDI-19, retained pUC13 as the vector.

3. Creation of an artificial DI SFV genome

(a) Strategy

At this stage of the project the sequences required by DI SFV genomes for propagation were not known, and it was suspected that, like DI Sindbis genomes, only the terminal sequences and part of the nsP1 coding region were needed (Levis *et al.*, 1986). In order to create an artificial DI SFV genome, it was therefore decided to centrally delete sequences from the full-length infectious clone pSP6-SFV4 (Liljeström *et al.*, 1991) to leave the non-coding terminal sequences and the 5' terminus of the nsP1 gene. It was not known if the genome size of a DI SFV was important for propagation as virions and so it was decided to produce a deleted molecule of between 2.0 and 2.5 kbp to match the size of pSFVDI-6 and previously cloned DI SFV genomes (Lehtovaara *et al.*, 1982).

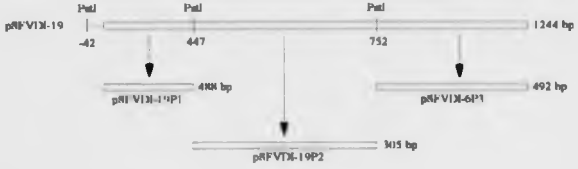


Figure 4.7 Diagrams of PstI sub-clones of pSFVDI-19 used for sequencing

DNA fragments are shown as linear segments, with the position of the PstI site of pUC13 indicated with respect to the first nucleotide of the SFV-derived sequences of the insert pSFVDI-19 was digested with PstI, and the 488 and 305 bp fragments isolated These were cloned into PstI-digested pBluescribe II KS^{+} to give pSFVDI-19P1 and pSFVDI-19P2 respectively pSFVDI-19P3 was made by religation of PstI-digested pSFVDI-19, and hence retained pUC13 as the vector

(b) Deletion of pSP6-SFV4

The restriction map of pSP6-SFV4 (Figure 4.8) indicated that a suitable internal deletion would be generated by using SalI to cleave the clone at the 5' terminus, and BstEII or NdeI to cleave at the 3' terminus. Figure 4.9a shows the consequence of single and double digestions with these enzymes. Because the SalI-NdeI digest did not produce the expected band pattern on an agarose gel, probably due to incomplete digestion, and the SalI-BstEII one did, the latter was used as the source of DNA for cloning. It was likely, because the cleavage points of these enzymes were known, that the 5.1 kbp band shown in Figure 4.9a comprised the sequences of interest, but it was unnecessary to purify this DNA since none of the others contained sequences capable of replication in bacteria. The digested plasmid was treated with the large fragment of *E.coli* DNA polymerase I to fill in the cohesive ends that had been generated, and this was religated and transformed into *E.coli* (TG2) cells. Figure 4.9b shows a plasmid preparation from one clone that was obtained. Here it was digested with NheI to give a three-band pattern indicating that only the terminal sequences of pSP6-SFV4 had been retained (see Figure 4.8). Digestion with SpeI ensured that linearisation was still possible: this enzyme is used to cleave the full-length clone at the 3' terminus of the insert such that it can be transcribed. Comparison of digests of pSFVDI-6 shows that the resulting clone, designated pSFVDI-SB1, was of a similar size, and by calculation the insert was 2.2 kbp in length. Further evidence that the central regions of pSP6-SFV4 had been deleted was indicated by the fact that pSFVDI-SB1 was not cleaved by EcoRI (data not shown): this enzyme cuts the full-length clone at four sites (see Figure 4.8).

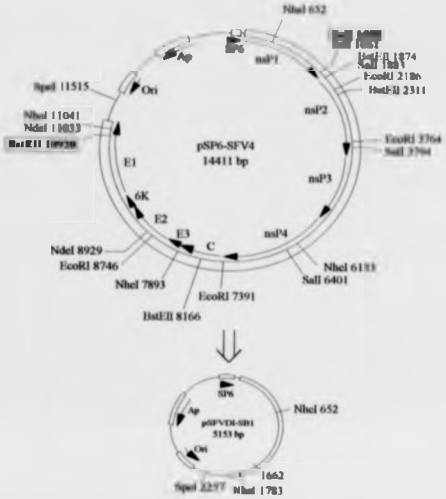


Figure 4.8 Construction of pSFVDI-SB1 from pSP6-SFV4

The diagram of pSP6-SFV4 is reproduced from Liljeström and Garoff (1991b), and shows the SFV coding regions contained within an SP6 expression vector (pPLH211) and relevant restriction sites. The vector is also cleaved by NheI - no information was available on the precise location of this site. To construct pSFVDI-SB1, the region between SalI 1658 and BstEII 10920 of pSP6-SFV4 (boxed) was deleted. Sticky ends were filled prior to ligation. The position of ligation in pSFVDI-SB1 is indicated as nucleotide 1662.

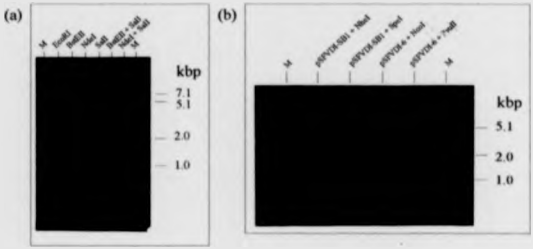


Figure 4.9 Restriction analysis of pSP6-SFV4 **(a)** and pSFVDI-SB1 **(b)**

pSP6-SFV4 (Liljeström *et al.*, 1991) was digested with a number of restriction enzymes to discover which gave suitable fragments for cloning. The EcoRI digest was included to confirm pSP6-SFV4 had not lost sequences during preparation: this digestion gives a characteristic 4-band pattern on an agarose gel. Most digestions gave the expected fragments as determined from the restriction map of pSP6-SFV4; the NdeI + SalI digestion was partial. DNA from the BstEII + SalI digestion was religated and transformed into bacteria. A clone was obtained (pSFVDI-SB1) of 5.2 kbp, which corresponded to the expected size of a SalI-BstEII deletion of pSP6-SFV4. Digestion with NheI gave three fragments of 1.8, 1.1 and 0.8 kbp, while SpeI cleaved the plasmid once **(b)**. Comparison to digestions of pSFVDI-6 showed the plasmids, and by calculation their respective SFV-derived sequences were of a similar size.

DISCUSSION

In a typical cloning experiment using blunt-ended PCR products, less than 1% of the colonies that were transformed were found to harbour recombinant plasmids. This was probably a consequence of the low efficiency with which the blunt-ended DNA ligated into the vector. In the cloning experiment shown in Figure 4.1, all the putative recombinants had inserted DNA and a quarter had the DNA sequence of interest, showing that the selection of recombinants was accurate and thus it was not necessary to find an alternative screening method. However, analysis of the termini of several cloned DNAs by sequencing showed that approximately 50% had lost nucleotides. This loss may have occurred in the process of ligation, or because some of the PCR products that were cloned did not have intact termini. The latter would have been possible if the synthetic oligonucleotide preparation used for PCR was heterogeneous, with some molecules lacking terminal nucleotides. Whatever the reason, in retrospect it may have been advantageous to design PCR primers that included unique restriction enzyme sites. These would have enabled the DNA produced on PCR amplification to be ligated using cohesive termini, which is more efficient than blunt-ended ligation and permits molecules with correct termini to be selected for in the process of ligation. However, in later experiments primers were used that incorporated features permitting RNA transcription and therefore contained many additional nucleotides. It was thought that the inclusion of more non-viral sequences would have reduced the sensitivity of reverse transcription and PCR amplification.

The first DI SFV clones that were obtained were found to have inserted DNA of 2.1 kbp, a size similar to that of previously cloned DI SFV genomes. However,

because restriction digests of these clones did not produce fragments of the same size, and some enzymes cleaved only once, it was probable that these clones did not possess extensive repeat regions (unlike previously cloned genomes). Tentative conclusions were made in the Results section of this chapter on the derivation of the cloned molecules from SFV RNA. These conclusions were confirmed by sequencing (see Chapter 5) and by propagation of RNA transcribed from the clones in the form of virus (see Chapter 7).

The eight clones designated pSFVDI-1 to 8 were all suspected to have the same inserted DNA. This suggests either that the 2.1 kbp PCR product that was cloned comprised just one species of DNA and hence the method of PCR amplification was selective for particular molecules, or that the DI SFV preparation from which the PCR product was derived was homogeneous. The latter is unlikely because DI SFV populations have been shown to be heterogeneous (Kääriäinen *et al.*, 1981, Barrett *et al.*, 1984a). It is likely, therefore, that the reason several identical clones were obtained from two separate preparations of DI SFV was that PCR preferentially amplified just one RNA species.

The PCR product that was generated from the mouse-protecting preparation DI SFV-p7 was shown in Chapter 3 to differ from that derived from the non-protecting DI SFV-p8 by having an additional 1.2 kbp species. Although no DI SFV genomes have been found to date of less than 2 kb in size, the evidence described in the Results section suggested that this was a true DI genome. For reasons described above for pSFVDI-6, the restriction analysis of the clone that was produced from this product (pSFVDI-19) suggested that there were no

extensive repeat regions, which are characteristic of previously cloned DI SFV genomes.

The modification of the termini of pSFVDI-6 and pSFVDI-19 used the clones themselves as templates for PCR amplification, because of limited starting material. It would have been desirable to amplify from the original RNA preparation to minimise the incorporation of errors into the template during amplification. However, as discussed in Chapter 5, it was unlikely that a few nucleotide differences from the RNA from which the clones were derived would be deleterious to the biological characteristics of the DI genome.

The two deletion mutants pSFVDI-1A2 and pSFVDI-M2 were made primarily to study interference and propagation of DI SFV. It was not known at the time if DI SFV genomes had a minimum size limit below which they would not be encapsidated. Fortunately pSFVDI-19, which is smaller than both these clones, was found to be propagated as virions, and the sub-clones were thus deemed to be suitable for further investigation. However, because the primary purpose of this project was to study mouse-protecting DI SFV genomes, and because these deleted forms of pSFVDI-6 were suspected to comprise non-protecting sequences, they were not investigated extensively.

The attempt at producing an artificial DI genome was made for the reasons described in the Introduction, and because it was not possible to propagate RNA transcripts of the putative DI SFV clones in tissue culture at the time. There was concern that this was a consequence of the clones not possessing correct termini. RNA transcripts from the full-length SFV clone, known to be propagated

efficiently as virus in tissue culture, possess only one non-viral nucleotide on the 5' terminus, and have a 70 base polyadenylation sequence at the 3' terminus (Liljestrom *et al.*, 1991). There was little information on the structure of the termini of DI SFV genomes, but it was probable that deletion of pSP6-SFV4 would generate a defective genome with termini consistent with those of a DI genome. Hence, if RNA transcribed from this deleted clone (pSFVDI-SB1) was not propagated as virions in tissue culture it would probably be because it lacked other sequences essential to DI SFV propagation.

CHAPTER 5

THE NUCLEOTIDE SEQUENCES OF CLONED DEFECTIVE RNAs OF SEMLIKI FOREST VIRUS

INTRODUCTION

The previous chapter described the construction of a number of DNA clones derived from the mouse-protecting virus preparation, DI SFV-p7. This chapter describes the nucleotide sequences of two of the clones, pSFVDI-6 and pSFVDI-19, which were used in subsequent transfection experiments. Comparisons are made with SFV RNA and with previously published DI SFV genomes.

The nucleotide sequences of pSFVDI-6 and pSFVDI-19 were determined primarily to establish the regions of SFV RNA from which they were derived and to learn more about the mechanisms involved in their generation. The primary structure of DI genomes in general contains the information required for replication and encapsidation by standard virus proteins, and by definition the information for interference and amplification. Because DI genomes are only a fraction of the size of the standard genome, these properties reside within a relatively small length of nucleic acid. By comparing nucleotide sequences of DI and standard virus, regions of the standard genome can be defined as recognition sites for the propagative proteins of the virus. The comparisons made in this chapter between the DI SFV clones and the SFV genome therefore demonstrate that DI genomes are useful tools for studying the molecular biology of the virus from which they are derived.

As stated previously, little information is available on the nucleotide sequences of DI SFV genomes. Lehtovaara *et al.* (1981, 1982) described the nucleotide sequences of two DI SFV RNAs, DI 301 and DI 309, from which the clones pKTH 301 and pKTH 309 were constructed. Because SFV RNA had not been completely sequenced at that time, it was not possible to determine how most of

the sequences of these RNAs had been derived from standard RNA. In this chapter, DI 301 and DI 309 are compared with pSFVDI-6 and pSFVDI-19 in an attempt to establish the nucleotide sequence content of DI SFV genomes in general

RESULTS

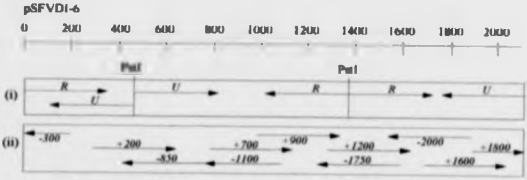
1. The nucleotide sequence of pSFVDI-6.

(a) Strategy for sequencing pSFVDI-6.

pSFVDI-6 was sequenced by the chain-termination method (Sanger *et al*., 1977) The sequencing strategy and the synthetic oligonucleotides used are shown in Figure 5.1 Sub-clones were made by digesting pSFVDI-6 with the restriction enzyme PstI, which generated fragments of approximately 800 and 900 bp These were ligated into pUC13 In addition, the digested pSFVDI-6 was religated, so that it contained the 500 nucleotides from the 5' terminus of the original clone These sub-clones permitted the majority of pSFVDI-6 to be sequenced by primer extension from within pUC13 In order to sequence both strands, and the remainder of the clone, several primers specific to pSFVDI-6 were used Primer extension from the oligonucleotide designated '*-2000*' (Figure 5.1) was only possible using a cycle sequencing method, probably because secondary structure in the 3' region of pSFVDI-6 was not removed at the temperature required for the standard sequencing method

(b) The nucleotide sequence of pSFVDI-6.

The nucleotide sequence of pSFVDI-6 is presented in Figure 5.2, with differences from the sequence of SFV (Garoff *et al*., 1980a, b, Riedel *et al*., 1982, Takkinen, 1986) indicated below the sequence The sequence given is that of the clone used for transfection experiments The original clone was modified by incorporation of



(i) Primers annealing to pUC13

U ('universal' primer) : 5'- GTTTTCCCAGTCACGAC - 3'
R ('reverse' primer) : 5'- AACAGCTATGACCATG - 3'

(ii) Primers annealing to pSFVDI-6

+200	5'-CGCACCTGGCTACCAAATT-3'	(bases 219-237)
-300	5'-CATTCTCCTGGAAGCGC-3'	(bases 304-287)
+700	5'-GGGCCGACGAGCAGGTG-3'	(bases 684-700)
-850	5'-GATCCCGGAACCCCAAAG-3'	(bases 854-837)
+900	5'-CCTCGTGACCAAACACGATC-3'	(bases 879-898)
-1100	5'-CCGAGGTTTAACAAGAGC-3'	(bases 1101-1084)
+1200	5'-CATCTGCACTGAAGTATGTC-3'	(bases 1182-1201)
+1600	5'-CATTCCACAGGGTAACT-3'	(bases 1595-1611)
-1750	5'-GCAGTGTCCAGGACAGGC-3'	(bases 1753-1729)
+1800	5'-GTGGAGCACCATATACCT-3'	(bases 1778-1794)
-2000	5'-GCGCAGGTCTTGTTGCGC-3'	(bases 2004-1987)

Figure 5.1 Strategy used to sequence pSFVDI-6

pSFVDI-6 was sequenced by the chain-termination method (Sanger *et al*., 1977) using primer extension from within pUC13 into the clone or sub-clone (i) or from within pSFVDI-6 itself (ii) Sub-clones were made from PstI-restricted fragments of pSFVDI-6 Arrows show the approximate number of nucleotides determined with each primer

pSFVDI-6 (2146 bases)

```
        10          20          30          40          50          60
ATGGCGGATG TGTGACATAC ACGACGCCAA AAGATTTTGT TCCAGCTCCT GCCACCTCCG
                                                                    G
        70          80          90         100         110         120
CTACGCGAGA GATTAACCAC CCACGATGGC CGCCAAAGTG CATGTTGATA TTGAGGCTGA

       130         140         150         160         170         180
CAGCCCATTC ATCAAGTCTT TGCAGAAGGC ATTTCCGTCG TTCGAGGTGG AGTCATTGCA

       190         200         210         220         230         240
GGTCACACCA AATGACCATG CAAATGCCAG AGCATTTTCG CACCTGGCTA CCAAATTGAT

       250         260         270         280         290         300
CGAGCAGGAG ACTGACAAAG ACACACTCAT CTTGGATATC GGCAGTGCGC CTTCCAGGAG

       310         320         330         340         350         360
AATGATGTCT ACGCACAAAT ACCACTGCGT ATGCCCTATG CGCAGCGCAG AAGACCCCGA

       370         380         390         400         410         420
AAGGCTCGTA TGCTACGCAA AGAAACTGGC AGCGGCCTCC GGGAAGGTGC TGGATAGAGA
        AU A
       430         440         450         460         470         480
GATCGCAGGG AAAATCACCG ACCTGCAGAC CGTCATGGCT ACGCCAGACG CTGAATCTCC
         A
       490         500         510         520         530         540
TACCTTTTGC CTGCATACAG ACGTCACGTG TCGTACGGCA GCCGAAGTGG CCGTATACCA

       550         560         570         580         590         600
GGACGTGTAT GCTGTACATG CACCAACATC GCTGTACCAT CAGGCGATGA AAGGTGTCAG

       610         620         630         640         650         660
AACGGCGTAT TGGATTGGGT TTGACACCAC CCCGTTTATG TTTGACGCGC TAGCAGGCGC

       670         680         690         700         710         720
GTATCCAACC TACGCCACAA ACTGGGCCGA CGAGCAGGTG TTACAGGCCA GGAACATAGG

       730         740         750         760         770         780
ACTGTGTGCA GCATCCTTGA CTGAGGGAAG ACTCGGCAAA CTGTCCATTC TCCGCAAGAA

       790         800         810         820         830         840
GCAATTGAAA CCTTGCGACA CAGTCATGTT CTCGGTAAGA CTACAGTAGT AGGAGTCTTT

       850         860         870         880         890         900
GGGGTTCCGG GATCAGGCAA GTCTGCTATT ATTAAGAGCC TCGTGACCAA ACACGATCTG
```

[continued overleaf]

```
       910         920         930         940         950         960
GTCACCAGCG GCAAGAAGGA GAATTGCCAG GAAATAGTCA ACGACGTGAA GAAGCACCGC
                          C           U
       970         980         990        1000        1010        1020
GGACTGGACA TCCAGGCAAA AACAGTGGAC TCCATCCTGC TAAACGGGTG TCGTCGTGCC
 |----------------|            X
      1030        1040        1050        1060        1070        1080
GTGGACATCC TATATGTGGA CGAGGCTTTC GCTTGCCATT CCGGTACTCT GCTGGCCCTA

      1090        1100        1110        1120        1130        1140
ATTGCTCTTG TTAAACCTCG GAGCAAAGTG GTGTTATGCG GAGACCCCAA GCAATGCGGA

      1150        1160        1170        1180        1190        1200
TTCTTCAATA TGATGCAGCT TAAGGTGAAC TTCAACCACA ACATCTGCAC TGAAGTATGT

      1210        1220        1230        1240        1250        1260
CATAAAAGTA TATCCAGACG TTGCACGCGT CCAGTCACGG CCATCGTGTC TACGTTGCAC

      1270        1280        1290        1300        1310        1320
TACGGAGGCA GGATGCGCAC GACCAACCCG TGCAACAAAC CCATAATCAT AGACACCACA
            A
      1330        1340        1350        1360        1370        1380
GGACAGACCA AGCCCAAGCC AGGAGACATC GTGTTAACAT GCTTCCGAGG CTGGGTAAAG
                                                              C
      1390        1400        1410        1420        1430        1440
CAGCTGCAGT TGGACTACCG TGGACACGAA GTCATGACAG CAGCAGCATC TCAGGGCCTC
                                          C
      1450        1460        1470        1480        1490        1500
ACCCGCAAAG GGTATACGCC GTAAGGCAGA AGGTGAATGA AAATCCCTTG TATGCCCCTG
           ⇧
      1510        1520        1530        1540        1550        1560
CGTCGGAGCA CGTGAATGTA CTGCTGACGC GCACTGAGGA TAGGCTGGTG TGGAAAACGC

      1570        1580        1590        1600        1610        1620
TGGCCGGCGA TCCCTGGATT AAGGTCCTAT CAAACATTCC ACAGGGTAAC TTTACGGCCA

      1630        1640        1650        1660        1670        1680
CATTGGAAGA ATGGCAAGAA GAACACGACA AAATAATGAA GGTGATTGAA GGACCGGCTG

      1690        1700        1710        1720        1730        1740
CGCCTGTGGA CGCGTTCCAG AACAAAGCGA ACGTGTGTTG GGCGAAAAGC CTGGTGCCTG

      1750        1760        1770        1780        1790        1800
TCCTGGACAC TGCCGGAATC AGATTGACAG CAGAGGAGTG GAGCACCATA TACCTCTATT
                                                       |----------
      1810        1820        1830        1840        1850        1860
ATAATCACTT GAACGAAAAC TGAAAAACAG AAAAAGTTAG GGTAAGCAAT GGCATTAATA
-------------|       A           U  X          G           G
```

[continued overleaf]

```
      1870        1880        1890        1900        1910        1920
TAGCAAGAAA ACCGAAAATA GAAAAAGTTA GGGTAGGCAA TGGCATTGAT ATAGCAAGAA
           TT      C     U  X
      1930        1940        1950        1960        1970        1980
AATTGAAAAC AGAAAAAGTT AGGGTAAGCA ATGGCATATA ACCATAACTG TATAACTTAT
                                                                  G
      1990        2000        2010        2020        2030        2040
AACAAAGCGC AACAAGACCT GCGCAATTGG CCCCGTGGTC CGCCTCACGA AACTCGGGGC
                                                      ⇧
      2050        2060        2070        2080        2090        2100
AACTCATATT GACACATTAA TTGGCAATAA TTGGAAGCTT ACATAAGCTT AATTCGACGA

      2110        2120        2130        2140
ATAATTGGAT TTTTATTTTA TTTTGCAATT GGTTTTTAAT ATTTCC
```

Figure 5.2 The nucleotide sequence of pSFVDI-6

Differences with respect to SFV RNA are indicated below the sequence ⇧ indicates the position of an additional base in SFV RNA in both cases this is a guanine X indicates the absence of a base in SFV RNA Base substitutions are also shown The underlined sequences from 963 to 978 and 1791 to 1814 cannot be assigned to SFV RNA The region from 1828 to 1879 is an imperfect repeat of nucleotides 1880 to 1930

a promoter for T3 RNA polymerase at the 5' terminus and the restriction enzyme site NcoI at the 3' terminus (see Chapter 4). The complete sequence of the insert in pUC13 has therefore the following additions to the sequence shown -

5' - GCA ATT AAC CCT CAC TAA AG ----
----GTA CCT T - 3'

Further, in the original clone there was an additional guanine between nucleotides 2029 and 2030, which is also contained in the sequence of SFV, and was lost in subsequent manipulations of the clone. pSFVDI-6 was one of eight clones that were shown to have inserts of 2.1 kbp, designated pSFVDI-1 to pSFVDI-8 (see Chapter 4). Of these clones, pSFVDI-1,7 and 8 were partly sequenced, and the 300 nucleotides at each terminus were shown to be identical to those of pSFVDI-6. This further supported the conclusion that the insert in pSFVDI-6 was derived from common sequences in the RNA extracted from tissue culture fluid, and therefore from common DI virus particles.

(c) Derivation of pSFVDI-6 from SFV RNA.

The derivation of pSFVDI-6 from the standard genome is shown in Figure 5.3, and is described in detail below. Note that some regions of pSFVDI-6 may have insertions or deletions with respect to SFV, and for this reason two regions described as homologous may not be exactly the same length. There are three regions that have extensive homology with SFV RNA. The first 817 nucleotides are homologous to the 5' terminus of SFV RNA, and therefore have the potential

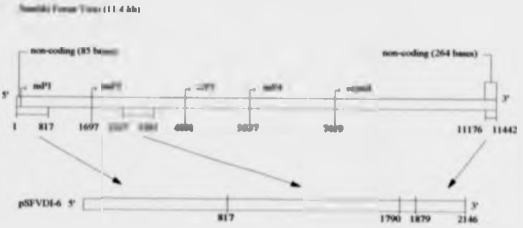


Figure 5.3 Comparison of pSFVDI-6 to SFV RNA

Arrows indicate the regions of SFV from which pSFVDI-6 was derived. For clarity, regions that cannot be assigned to SFV RNA (see Figure 5.2 and accompanying text) have been omitted. Because of overlapping sequences, the numbers shown below pSFVDI-6 refer to the 3' extents of each region of homology with SFV RNA.

to encode part of the first non-structural protein (nsP1). A protein of molecular weight (M_r) 27,000 produced by *in vitro* translation of RNA transcribed from pSFVDI-6 is consistent with this conclusion (see Chapter 6). Nucleotides 816 and 817 also appear in the 147 nucleotide region from 816 to 962 which are homologous to 2227 to 2373 of SFV RNA, so that the junction between the first two regions of homology with SFV RNA cannot be determined precisely. The 812 nucleotides from 979 to 1790 are homologous with 2391 to 3202 of SFV RNA, which are within the nsP2 coding region. The 16 nucleotides from 963 to 978 cannot be assigned with any certainty to SFV RNA, and the same is true for the 24 nucleotides from 1791 to 1814. The derivation of the 13 nucleotides from 1815 to 1827 is not certain, but is likely to be from nucleotides 11220 to 11232 of SFV RNA which also has the sequence [5'-G-4A-NN-G-5A-3']. The 52 nucleotide region from 1828 to 1879 is an imperfect repeat (86% homology) of nucleotides 1880 to 1930, which correspond to nucleotides 11176 to 11225 of SFV RNA. Because there is a 28 nucleotide imperfect repeat in SFV RNA itself (11175 to 11201 and 11225 to 11252), it is possible that the 28 nucleotides from 1828 to 1855 of pSFVDI-6 were originally derived from the sequence from 11225 to 11252 of SFV RNA, with which they have 100% homology. Finally the 267 nucleotides at the 3' terminus of pSFVDI-6, from 1880 to 2146 correspond to the non-coding 3' terminus of SFV RNA.

(d) Comparison of pSFVDI-6 with other DI SFV clones.

Figures 5.4a and 5.4b show comparisons of pSFVDI-6 with DI 301 and DI 309 (Lehtovaara *et al.*, 1981, 1982). These RNA sequences were determined from the DNA clones pKTH 301 and pKTH 309, both of which lacked the 5' termini of their

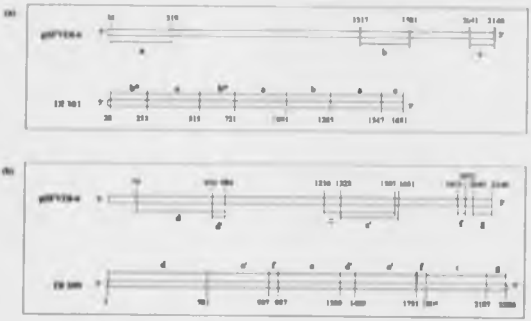


Figure 5.4 Comparison of pSFVDI-6 with DI 301 and DI 309

pSFVDI-6 and the DI SFV RNAs (Lehtovaara *et al.*, 1981, 1982) are represented as linear sequence blocks. DI 301 and DI 309 have been simplified to show only extensive regions of homology with pSFVDI-6, which are labelled a-c in **(a)** and d-g in **(b)**. Because some regions of homology overlap, only the nucleotides at the 3' termini of each region of the DI RNAs are numbered. Approximately 200 nucleotides from upstream of the 5' terminus of DI 301 have not been determined. In **(a)** the two regions labelled b* are deleted forms of region b, lacking the 59 nucleotides which correspond to nucleotides 1447 to 1505 of the pSFVDI-6 genome.

respective RNAs the 5' terminal nucleotides of DI 309 were determined from cDNA. Like the DI RNA from which pSFVDI-6 was derived, DI 301 and DI 309 were extracted from DI SFV produced by serial undiluted passage of SFV in BHK-21 cells. All regions of both RNAs are contained within pSFVDI-6 and because they contain several repeated regions they possess approximately one half of the sequences contained in the pSFVDI-6 genome. This suggests that much of pSFVDI-6 is unnecessary for propagation in tissue culture: analysis of deletion mutants (see Chapter 7) supports this conclusion. DI 301 contains a triplicated linear repeat of the regions designated a and b. Region 'a' corresponds to the 284 nucleotide region of pSFVDI-6 derived from the 5' terminus of SFV RNA. Not all of the 5' non-coding 85 nucleotides of SFV RNA are represented in DI 301, probably because approximately 200 nucleotides from the 5' terminus were not determined. Region 'b' is homologous to the 265 nucleotides from 1317 to 1581, which correspond to the nsP2 coding region of SFV RNA, from nucleotides 2728 to 2993 of SFV RNA. The two regions designated b* are deleted forms of region b, lacking the 59 nucleotides of pSFVDI-6 from 1447 to 1505. As noted by Lehtovaara *et al.* (1981), the deleted nucleotides present in region b have a degree of inverted complementarity, suggesting that this RNA may form base-paired secondary structure in this region. Region 'c' comprises 106 nucleotides from the 3' terminus of pSFVDI-6. This corresponds to the 3' terminus of SFV RNA, and suggests that not all of the 3' untranslated region (264 nucleotides) is necessary for DI virus propagation.

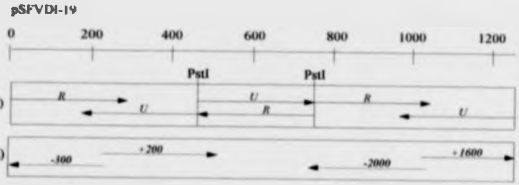
DI 309 (Figure 5.4b) is composed of similar regions of homology with pSFVDI-6 and hence SFV RNA. Region 'd' comprises 581 nucleotides adjacent to the 5' terminus of pSFVDI-6, and has a 26 nucleotide deletion of this region, from 138 to

163 of pSFVDI-6 (not shown in Figure 5.4b). The 69 nucleotides comprising region d' are also homologous to part of region d, corresponding to nucleotides 616 to 684 of pSFVDI-6. The 382 and 329 nucleotide regions designated e and e' are homologous to the central region of pSFVDI-6, from nucleotides 1216 to 1597 and 1323 to 1651 respectively. These correspond to part of the *nsP2* coding region of SFV RNA, from nucleotides 2627 to 3009 and 2734 to 3063. The sequence in the 3' region of pSFVDI-6 designated 'f' is represented twice in DI 309; in both cases it is flanked by sequences that are not contiguous with it in the SFV genome.

2. The nucleotide sequence of pSFVDI-19

(a) Strategy used to sequence pSFVDI-19.

pSFVDI-19 was sequenced by the chain-termination method (Sanger *et al.*, 1977). The sequencing strategy and the synthetic oligonucleotides used are shown in Figure 5.5. Three sub-clones were made. pSFVDI-19 was digested with PstI to give fragments of approximately 500 and 300 bp, which were ligated into pUC13, and the digested pSFVDI-19 was religated so that it contained only the 500 bp from the 5' terminus. These sub-clones permitted the complete sequence of pSFVDI-19 to be obtained by primer extension from within pUC13. In order to sequence both strands, primers specific for pSFVDI-19 were used. Primer extension from the oligonucleotide designated *'-2000'* was only possible using a cycle sequencing method, for reasons stated previously for the sequencing of pSFVDI-6.



(i) Primers annealing to pUC13

U ('universal' primer) : 5'-GTTTTCCCAGTCACGAC-3'
R ('reverse' primer) : 5'-AACAGCTATGACCATG-3'

(ii) Primers annealing to pSFVDI-19

+200 : 5'-CGCACCTGGCTACCAAATT-3' (bases 219-237)
-300 : 5'-CATTCTCCTGGAAGGCGC-3' (bases 304-287)
+1600: 5'-CATTCCACAGGGTAACT-3' (bases 959-975)
-2000: 5'-GCGCAGGTCTTGTTGCGC-3' (bases 1101-1084)

Figure 5.5 Strategy used to sequence pSFVDI-19

pSFVDI-19 was sequenced by the chain-termination method using primer extension from within pUC13 into the clone or subclone (i) or from within pSFVDI-19 itself (ii). Subclones were made from PstI-restricted fragments of pSFVDI-19. Arrows show the approximate number of nucleotides determined with each primer. Primer designations are derived from the approximate position of annealing to pSFVDI-6 (see Figure 5.1).

(b) The nucleotide sequence of pSFVDI-19.

Figure 5.6 shows the nucleotide sequence of pSFVDI-19. Like pSFVDI-6, the original clone from which pSFVDI-19 was derived did not possess the promoter for T3 RNA polymerase, or a restriction enzyme site to permit run-off transcription. The sequence given is therefore that of the modified clone, and the complete insert in pUC13 has the additional sequences described previously for pSFVDI-6. Base 22, given as thymine, was cytosine in the original clone, and inadvertently altered in subsequent manipulations of the clone. There are open reading frames and RNA transcribed from pSFVDI-19 produced a protein of M_r 25,000 on *in vitro* translation (see Chapter 6).

(c) Derivation of pSFVDI-19 from SFV RNA.

pSFVDI-19 is composed of three regions which have extensive homology to regions of SFV RNA (Figure 5.7). Nucleotides 1 to 576 correspond to the same region of SFV, the 401 nucleotides from 576 to 976 with nucleotides 2623 to 3024 (base 576 is also present in this region of homology) and the 268 nucleotides from 977 to 1244 with 11176 to 11442 of SFV RNA. The 5' terminus of pSFVDI-19 is therefore derived from the nsP1 coding region of SFV RNA, and includes the 85 non-coding nucleotides from the 5' terminus of SFV RNA. The central region of pSFVDI-19 is derived from the nsP2 coding region of SFV RNA, and the 3' terminus from the non-coding 3' terminus of SFV RNA, containing all 264 nucleotides of this region.

pSFVDI-19 (1244 bases)

```
        10          20          30          40          50          60
ATGGCGGATG TGTGACATAC ATGACGCCAA AAGATTTTGT TCCAGCTCCT GCCACCTCCG
                    C                                             G
        70          80          90         100         110         120
CTACGCGAGA GATTAACCAC CCACGATGGC CGCCAAAGTG CATGTTGATA TTGAGGCTGA

       130         140         150         160         170         180
CAGCCCATTC ATCAAGTCTT TGCAGAAGGC ATTTCCGTCG TTCGAGGTGG AGTCATTGCA

       190         200         210         220         230         240
GGTCACACCA AATGACCATG CAAATGCCAG AGCATTTTCG CACCTGGCTA CCAAATTGAT

       250         260         270         280         290         300
CGAGCAGGAG ACTGACAAAG ACACACTCAT CTTGGATATC GGCAGTGCGC CTTCCAGGAG

       310         320         330         340         350         360
AATGATGTCT ACGCACAAAT ACCACTGCGT ATGCCCTATG CGCAGCGCAG AAGACCCCGA

       370         380         390         400         410         420
AAGGCTCGTA TGCTACGCAA AGAAACTGGC AGCGGCCTCC GGGAAGGTGC TGGATAGAGA
        AT  A
       430         440         450         460         470         480
GATCGCAGGG AAAATCACCG ACCTGCAGAC CGTCATGGCT ACGCCAGACG CTGAATCTCC

       490         500         510         520         530         540
TACCTTTTGC CTGTATACAG ACGTCACGTG TCGTACGGCA GCCGAAGTGG CCGTATACCA
             C
       550         560         570         580         590         600
GGACGTGTAT GCTGTACATG CACCAACATC GCTGTATCCA GACGTTGCAC GCGTCCAGTC

       610         620         630         640         650         660
ACGGCCATCG TGTCTACGTT GCACTACGGA GGCAGGATGC GCACGACCAA CCCGTGCAGC
                                        A                       A
       670         680         690         700         710         720
AAACCCATAA TCATAGACAC CACAGGACAG ACCAAGCCCA AGCCAGGAGA CATCGTGTTA

       730         740         750         760         770         780
ACATGCTTCC GAGGCTGGGT AAAGCAGCTG CAGTTGGACT ACCGTGGACA CGAAGTCATG
                    C
       790         800         810         820         830         840
ACAGCAGCAG CATCTCAGGG CCTCACCCGC AAAGGGTATA CGCCGTAAGG CAGAAGGTGA
                                            ⇧
       850         860         870         880         890         900
ATGAAAATCC CTTGTATGCC CCTGCGTCAG AGCACGTGAA TGTACTGCTG ACGCGCACTG

       910         920         930         940         950         960
AGGATAGGCT GGTGTGGAAA ACGCTGGCCG GCGATCCCTG GATTAAGGTC CTATCAAACA

       970         980         990        1000        1010        1020
TTCCACAGGG TAACTTAGAA AAAGTTAGGG TAGGCAATGG CATTGATATA GCAAGAAAAT
             U   X
```

[continued overleaf]

```
      1030        1040        1050        1060        1070        1080
TGAAAACAGA AAAAGTTAGG GTAAGCAATG GCATATAACC ATAACTGTAT AACTTATAAC
                                                              G
      1090        1100        1110        1120        1130        1140
AAAGCGCAAC AAGACCTGCG CAATTGGCCC CGTGGTCCGC CTCACGGAAA CTCGGGGCAA

      1150        1160        1170        1180        1190        1200
CTCATATTGA CACGTTAATT GGCAATAATT GGAAGCTTAC ATAAGCTTAA TTCGACGAAT
            A
      1210        1220        1230        1240
AATTGGATTT TTATTTTATT TTGCAATTGG TTTTTAATAT TTCC
```

Figure 5.6 The nucleotide sequence of pSFVDI-19

Differences with respect to SFV RNA are shown below the sequence X indicates the absence and U the presence of a nucleotide in SFV RNA

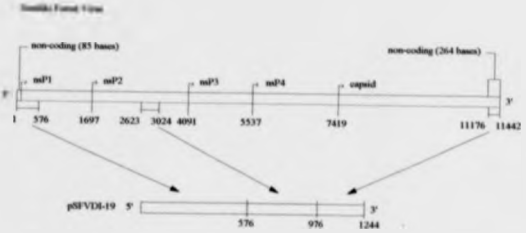


Figure 5.7 Comparison of pSFVDI-19 to SFV RNA

Arrows indicate the regions of SFV RNA from which pSFVDI-19 was derived. The numbers shown below pSFVDI-19 refer to the nucleotides at the 3' terminus of each region of homology

3. Comparison of pSFVDI-6 and pSFVDI-19.

Figure 5.8 shows regions of homology between pSFVDI-6 and pSFVDI-19. All sequences contained within pSFVDI-19 are represented in pSFVDI-6. Nucleotide differences of pSFVDI-19 with SFV RNA shown in Figure 5.6 are the same as those for pSFVDI-6 (Figure 5.2) except for the two nucleotides known to have altered as a consequence of modifications to the two clones. The sequence on the 3' side of the junction between the central and 3' region is the same for both cloned DNAs. While there are no other common sequences on either side of the junctions, the two bases 5'- thymine-adenine-3' are located at all the junction points of SFV RNA sequences in both pSFVDI-6 and pSFVDI-19. The significance of this is not known. As mentioned previously, the three regions of homology with SFV RNA of both pSFVDI-6 and pSFVDI-19 are derived from the 5' nsP1 coding region, the nsP2 coding region, and the 3' non-coding terminus, suggesting that these DI sequences were generated by a common mechanism. The central regions of these clones, unlike the terminal regions, were not selected for by the primers used to isolate these DI virus sequences originally. It seems likely, therefore, that the central regions are necessary for some function of these DI sequences.

4. Secondary structure prediction

The nucleotide sequences of pSFVDI-6 and pSFVDI-19 were analysed by computer for regions that may be involved in RNA folding. However, the results of this analysis were ambiguous, to the extent that it was not considered worthwhile

to present them here. In addition, pSFVDI-6 and pSFVDI-19 were too large to be analysed by the computer software in their entirety.

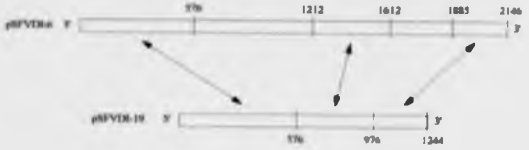


Figure 5.8 Comparison of pSFVDI-6 and pSFVDI-19

Arrows indicate regions of homology between pSFVDI-6 and pSFVDI-19.
Numbers refer to the nucleotides at the 3' termini of each region of homology.

DISCUSSION

Initially the justification for sequencing pSFVDI-6 and pSFVDI-19 was based on analyses of insert size and restriction sites (see Chapter 4). This indicated that the clones were derived from SFV sequences and were not merely artefacts of the methodology employed for cloning. Because the polymerase chain reaction was used for the amplification of DI SFV RNA and for modification of the original clones, it was possible that errors were introduced into the final DNA copies that were cloned. Although it would have been necessary to sequence more than one clone completely to establish the exact sequence of the original RNA template, for the purposes of this project it was more important to know the exact sequence of the DNA used for transcription and subsequent transfection. Hence DI virus populations generated from the clones could be defined on the basis of sequence content.

It is possible that the original RNA template from which the clones were derived also possessed other sequences not detected by RT-PCR amplification. It is known that the DI genomes of SFV are polyadenylated (Kennedy, 1976). In addition, three out of six DI genomes of Sindbis virus were found to have a structure contiguous with their 5' terminus that was almost identical to part (nucleotides 10-75) of the cellular rat $tRNA^{asp}$ (Monroe and Schlesinger, 1983; Tsiang *et al.*, 1985). These cellular sequences did not confer a competitive advantage over DI genomes without them. Whether DI SFV genomes have similar associated structures is not known. RNA transcripts of pSFVDI-6 and pSFVDI-19 proved to have all the necessary sequences required for propagation as virions (Chapter 7) and it was therefore not important to analyse the original templates further.

The two cloned sequences, pSFVDI-6 and pSFVDI-19 differed from previously isolated DI SFV genomes in having no extensive repeats or sequence rearrangements However, both were derived from the same regions of the SFV genome, suggesting a common requirement for these DI genomes The mismatches of the DI genomes with the published SFV sequence may have arisen *de novo*, or featured in the SFV RNA from which they were generated The deletions and insertions probably arose either during propagation of the defective RNAs, or in subsequent manipulations *in vitro* because their presence in the virion RNA would cause frame-shifts that would be likely to prevent the correct translation required for the production essential propagative proteins

Because the entire sequence of pSFVDI-19 is contained within pSFVDI-6, it is possible that the former was derived from the latter However, it is equally likely that both molecules were generated *de novo* from the standard genome The viral replicase may have a limited fidelity and jump from one region of the standard genome to another This jumping may be aided by secondary structure features of the standard genome, such as the circularisation of the molecule (Hsu *et al*., 1974; Frey *et al*., 1979) It is possible that a large range of molecules are produced by the viral replicase, and only those possessing sequences favouring amplification become established pSFVDI-6 was shown to have two regions of 16 and 24 nucleotides that could not be assigned to SFV RNA These regions may have been derived from cellular RNA species, or from an SFV RNA which differed in these regions from the published sequence The 52-nucleotide reiterated sequence of pSFVDI-6 derived from a region of SFV RNA that is adenine-rich and has the potential to base-pair with the thymine-rich 3' terminus This suggests that

secondary structure may be involved in the generation of this repeated sequence from standard RNA

It is clear that the mechanism by which the pSFVDI genomes were generated is less complicated than that which produced the repeated and rearranged sequences of the pKTH clones. Both DI SFV sequences described in this chapter were shown to be derived from the same regions of SFV RNA as pKTH 301 and pKTH 309. This suggests further that the minimum requirement of DI SFV for propagation by standard virus is the possession of parts of the terminal and naP2 sequences of SFV RNA. However, comparison of these clones indicates that approximately 70% of pSFVDI-6 and 45% of pSFVDI-19 is superfluous to the sequence requirement of these DI SFV molecules. It is possible that DI genomes have a minimum size, below which they cannot be packaged into virions. This implies that in addition to the sequences required for replication and encapsidation there need to be spacer sequences for viability. In the case of the pKTH clones a minimum size limit would be achieved by the possession of repeat sequences. It may be that while the initial structure of these molecules is determined by viral replicase errors, for the DI genomes to achieve sufficient size and hence be propagated as virions, polymerisation events must occur. However, another reason for the possession of repeated regions could be that they confer some advantage to the DI genome. For example, the possession of multiple replication initiation sites may result in the DI genome being amplified in preference to one with a only a single site.

Besides the naP2-3' junction of pSFVDI-6 and pSFVDI-19 which is the same for both clones, the sequences at the junction points of SFV RNA regions of these and the pKTH clones are different. It is therefore not possible to define a sequence

motif which could be responsible for a mechanism of DI genome generation. It is likely, therefore, that the initial putative replicase jumping that results in deletions of the standard genome occurs between juxtaposed secondary structure domains rather than between particular sequence motifs.

The conservation of a region that is derived from the nsP2 gene of SFV in both pSFVDI and pKTH clones suggests it has a *cis*-acting function necessary for the propagation of the DI RNA. The putative base-paired secondary structure within this region of pKTH 301 (designated 'b' in Figure 5.4a) may be involved in this function. Liljeström and Garoff (1991b) showed that deletion of the majority of the non-structural coding sequences of the infectious full-length SFV clone, pSP6-SFV4, produced a molecule whose RNA transcripts were replicated but not encapsidated. It seems likely, therefore, that this region of the nsP2 gene contains a recognition signal for capsid proteins. Jalenko and Soderlund (1985) replaced the late gene region of SV40 virus with the repeating units of pKTH 301. They showed that superinfection of monkey kidney cells harbouring the recombinant SV40 clone with SFV resulted in a reduction of the packaging efficiency of SFV RNA. They concluded that the terminal regions of pKTH 301 were required for efficient replication and the central repeating units (containing part of the nsP2 gene) for encapsidation.

The identification of regulatory sequences in the DI genomes of Sindbis virus appears to conflict with the above conclusions on the location of a packaging signal. It was shown that only the sequences that comprised the 162 nucleotide region at the 5' terminus and the 19 nucleotide region of the 3' terminus were necessary for replication and packaging of cloned DI Sindbis genomes (Levis *et al.*,

1986) However, more reliable evidence was provided by Weiss *et al.* (1989), who used capsid protein binding studies to identify a region from nucleotides 746 to 1226 (within the nsP1 gene) of Sindbis virus RNA that was required for packaging in tissue culture. Because Sindbis virus and SFV are closely related, it seems unlikely that the location of the encapsidation region would differ significantly. It will be necessary to use studies similar to those used by Weiss *et al.* (1989) to irrefutably define packaging domains of SFV RNA.

CHAPTER 6

TRANSCRIPTION AND TRANSLATION OF CLONED DEFECTIVE RNAs OF SEMLIKI FOREST VIRUS

INTRODUCTION

This chapter describes the RNA transcripts generated from the cloned defective SFV genomes described in Chapter 4 and directly from PCR products. In addition, the *in vitro* translation of RNA transcripts from pSFVDI-6 and pSFVDI-19 is described.

As shown in Chapter 4, the clones pSFVDI-6 and pSFVDI-19 and the sub-clone pSFVDI-M2 contained a promoter for T3 RNA polymerase directly upstream of their SFV-derived sequences and the restriction enzyme site for NcoI directly downstream, permitting run-off transcription. Transcripts produced from these clones were analysed before transfection into BHK-21 cells to ensure they had not degraded and that they matched the size of their respective templates. It was suspected that these RNA transcripts would be propagated more efficiently as virions in tissue culture if they possessed 5'-caps. Liljeström *et al.* (1991) showed that RNA transcripts of pSP6-SFV4 that were capped had an infectivity titre in BHK-21 cells approximately 100-fold greater than those without. It was not known if these results were applicable to the propagation as virions of defective genomes, but as a precaution, the first transfection experiments used capped RNAs. However, subsequent experiments suggested that uncapped RNA transcripts were propagated as virions just as efficiently (data not shown).

In a number of experiments RNA transcripts were generated directly from PCR products. As stated in Chapter 4, it was advantageous to determine if mouse-protecting DI SFV sequences were amplified by RT-PCR before screening individual clones for the possession of such sequences. As a first step to achieving

this, PCR products were generated from DI SFV-p7 using the primers *5'SFV-T3* and *3'SFV-Neo*. Hence a promoter for T3 RNA polymerase promoter was incorporated at the 5' termini of these PCR products, permitting them to be used as templates for RNA transcription. Because these primers possessed many non-SFV nucleotides, it was necessary to ensure that their use in the amplification from DI SFV RNA generated DNA products similar to those produced using the primers *5'SFV* and *3'SFV*. This chapter compares the PCR products derived from DI SFV-p7 using these different primer pairs, and shows the RNA that was transcribed from those PCR products containing a T3 RNA polymerase promoter.

The sub-clone pSFVDI-1A2, which was constructed by deleting internal sequences from the unmodified version of pSFVDI-6, was retained in the pBluescribe II KS$^+$ vector (see Chapter 4). RNA transcribed from this plasmid also included sequences derived from the vector. To modify the clone, RT-PCR was used to copy the DI SFV-derived sequences with the primers *5'SFV-T3* and *3'SFV-Neo*. This chapter describes the PCR products that were generated in this way, and the RNAs that were transcribed from them.

The final part of this chapter consists of an analysis of the polypeptides produced from RNA transcripts of pSFVDI-6 and pSFVDI-19 by *in vitro* translation. Open reading frames of the two clones were determined from their nucleotide sequences, and from these it was possible to speculate on which regions of the sequences had been translated. The nature and significance of these proteins are discussed.

RESULTS

1. Transcription of DI SFV clones

For clarity, RNA transcripts have been given the prefix 'r' (e.g. 'rSFVDI-19')

(a) Analysis of RNA transcripts

Products of RNA transcription were treated with either DNAse I or RNAse A to ensure that they were indeed RNA. Only the product treated with DNAse I was undegraded (data not shown). As stated in the Introduction, the first transfection experiments used capped RNA transcripts. Because these could not be distinguished from uncapped transcripts on an agarose gel, the results shown below are applicable to both.

RNA transcripts were analysed on agarose gels following denaturation with glyoxal to determine their size and integrity, and to permit subsequent northern transfer. Northern blot analysis of transcripts was not performed routinely for reasons of practicality, but was used for preliminary experiments to determine that the RNA transcripts did not contain minor species that were not detected by the usual methods of analysis. In most experiments, it was only necessary to determine that transcripts were intact before transfection. In these cases, RNA transcripts were analysed by standard agarose gel electrophoresis, as used for the analysis of DNA. RNA migrated at approximately twice the rate of DNA in this system, and it was therefore possible to estimate size by comparison with DNA markers.

It was deemed unnecessary to quantitate accurately the RNA produced in each transcription reaction. However, an estimate was made in order to standardise subsequent transfection experiments with respect to the quantity of input RNA. Each constituent species of the RNA ladder, in the volume that was electrophoresed, comprised approximately 0.5 µg RNA (Gibco BRL, Product specifications). Following electrophoresis, a visual comparison was made of the intensities of banded RNA transcripts to the banded RNA of the size marker. From this it was estimated that 1µg RNA per 1 µl was produced from the DNA template in a typical transcription reaction. There was no difference between the quantity of RNA produced in a transcription reaction that included a cap analogue and one that did not.

(b) Analysis of RNA transcripts from pSFVDI-6 and pSFVDI-19

Figure 6.1 shows an analysis by agarose gel electrophoresis and northern blotting of transcripts produced from pSFVDI-6 and pSFVDI-19 with T3 RNA polymerase. The respective transcripts are 2.1 and 1.2 kb in size, which corresponds to the size of the SFV-derived sequences of the clones. In Figure 6.1b, the low intensity bands of 3.9 kbp and 4.8 kbp in lanes 1 and 2 comprise template DNA which had not been removed at this stage. The origin of other low intensity bands is not known: these do not appear on an agarose gel following denaturation with glyoxal (Figure 6.1a), but this may be because the RNA is not stained as efficiently in this gel system.

The northern blot analysis shown in Figure 6.1c used [α-^{32}P]UTP-labelled RNA transcripts as a hybridisation probe. These were negative-strand transcripts, which

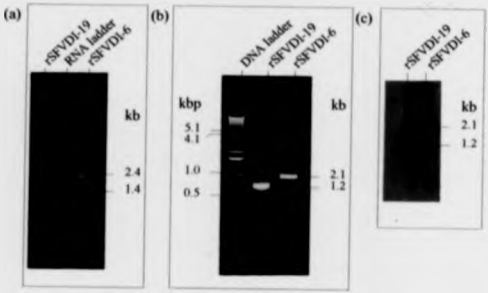


Figure 6.1 RNA transcripts from pSFVDI-6 and pSFVDI-19

RNA transcripts were analysed either following denaturation with glyoxal **(a)** or by standard agarose gel electrophoresis **(b)**. In **(a)**, the RNAs transcribed from pSFVDI-6 or pSFVDI-19 were 2.1 kb and 1.2 kb in size respectively. **(b)** shows that these transcripts migrate at approximately twice the rate of the DNA size marker, and is included because it exemplifies the method that was frequently used for estimating the size of RNA transcripts and establishing that they were undegraded. **(c)** shows a northern blot analysis of RNA transcripts produced from pSFVDI-6 and pSFVDI-19. By comparison with unlabelled size markers, the RNAs were determined to be 1.2 kb and 2.1 kb in size. No other RNA species were detected.

were produced from EcoRI-digested pSFVDI-7 using T7 RNA polymerase (pSFVDI-7 is described in Chapter 4: it comprises all the sequences of pSFVDI-6 inserted into the SmaI site of pBluescribe II KS+ in the 3'-5' orientation). Because this was a more sensitive technique for detecting RNA than staining with ethidium bromide, and since no other RNA species were detected, this analysis confirmed that the RNA transcripts were homogeneous with respect to size. This also suggested that the low intensity bands described above for Figure 6.1b did not comprise DI SFV sequences.

(c) Analysis of RNA transcripts from PCR products

Figure 6.2a shows analyses of the PCR products that were obtained from the mouse-protecting preparation DI SFV-p7, using the primers *5'SFV-73* and *3'SFV-Nco*. Comparison of these products with those obtained using the primers *5'SFV* and *3'SFV* shows that they are essentially the same, with a predominant DNA species of 1.2 kbp in size, and minor species of 1.6 and 2.1 kbp. The 0.6 kbp DNA present in the PCR product generated with the *5'SFV-3'SFV* primers is not present in the other. As discussed in Chapter 3, this DNA species was likely to have resulted from non-specific hybridisation by these primers.

Figure 6.2b shows an analysis of DNA produced by PCR amplification of pSFVDI-1A2 using the primers *5'SFV-73* and *3'SFV-Nco*. The sole product is 1.3 kbp, which corresponds to the size of the SFV-derived sequences in pSFVDI-1A2 (see Chapter 4). As positive controls for PCR, DNA was amplified from pSFVDI-M2 and pSFVDI-6. In both cases the predominant product corresponds in size to the SFV-derived sequences of the respective clones.

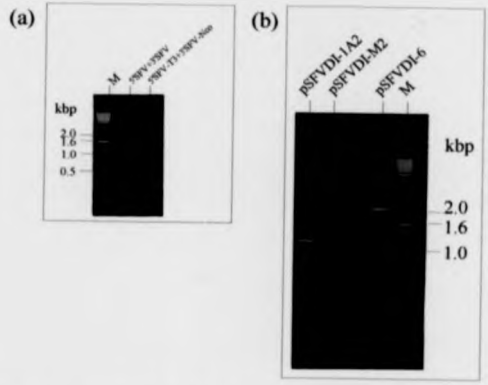


Figure 6.2 Agarose gel profile of PCR products

In **(a)** RNA was extracted from the mouse-protecting preparation, DI SFV-p7, and amplified by RT-PCR using the primer pairs *5'SFV* + *3'SFV* or *5'SFV-T3* + *3'SFV-Nco*. The main PCR products in both cases were DNA species of 1.2, 1.6 and 2.1 kbp. Amplification with the first primer pair yielded an additional product of 0.6 kbp.

In **(b)** PCR was used to amplify SFV-derived sequences from cloned DNA using the primers *5'SFV-T3* + *3'SFV-Nco*. The DNA amplified from pSFVDI-1A2, pSFVDI-M2 and pSFVDI-6 was 1.3, 1.7 and 2.1 kbp respectively.

Figure 6.3 shows a comparison of RNA transcripts derived from the PCR products described above. In Figure 6.3a, RNA transcribed from the (DI SFV-p7)-derived PCR product was shown to consist of two species, one of which co-migrated with the 2.1 kb RNA from pSFVDI-6, and another that was estimated to be 1.2 kb in size. Comparison with RNA transcribed from pSFVDI-19 confirmed that the latter was a 1.2 kb species (data not shown). The RNA transcribed from pSFVDI-M2 was approximately 1.7 kb in size and this was confirmed by agarose gel analysis after denaturation with glyoxal (data not shown). Figure 6.3b shows a comparison of transcripts from pSFVDI-M2 and pSFVDI-1A2. This was included to illustrate that there were similar quantities of RNA in both these preparations, as determined from their band intensities. By making this comparison, it was possible to ensure that similar quantities of RNA were used in a subsequent transfection experiment that compared the propagation of the two RNAs as virions (see Chapter 7).

Figure 6.3c shows a comparison of transcripts from pSFVDI-6 and the artificially-constructed clone, pSFVDI-SB1 (see Chapter 4). To obtain RNA transcripts, pSFVDI-SB1 was linearised with SpeI and transcribed with SP6 RNA polymerase. In both cases, the size of the RNA transcripts was 2.1 kb, which corresponds to those of the respective cloned DNAs. Also, both banded RNA transcripts were of a similar intensity, suggesting that similar quantities of each were produced in the transcription reactions.

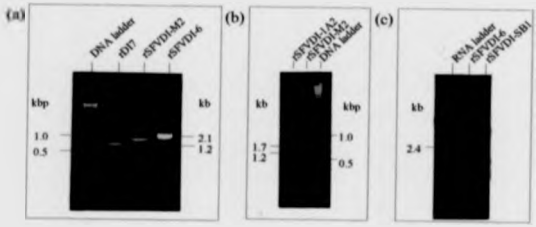


Figure 6.3 Further analysis of RNA transcripts

(a) and **(b)** show agarose gel profiles of RNA transcripts produced from PCR products. In lane 2 of **(a)**, RNA is shown that was transcribed from the PCR product derived from DI SFV-p7 (designated rDI7). Two predominant species were detected, which were estimated by comparison to a DNA size marker to be 1.2 and 2.1 kb. Transcription from pSFVDI-M2 and pSFVDI-6 gave products of 1.7 and 2.1 kb respectively. In **(b)** RNAs of 1.2 kb and 1.7 kb transcribed from the pSFVDI-1A2 PCR product and pSFVDI-M2 respectively are shown.

(c) shows an agarose gel profile of RNA transcripts, following denaturation with glyoxal. RNAs transcribed from pSFVDI-6 and pSFVDI-SB1 were both 2.1 kb.

2. *In vitro* translation of RNA transcripts from pSFVDI-6 and pSFVDI-19

Figure 6.4 shows open reading frames (ORFs) of greater than 200 nucleotides in pSFVDI-6 and pSFVDI-19. These were determined from the sequences of these two clones, which are described in Chapter 5. Figure 6.5 shows the products of translation from the RNA transcripts of pSFVDI-6 and pSFVDI-19. The protein of approximately M_r 27,000 from pSFVDI-6 RNA was probably encoded from the ORF that extends from nucleotides 86 to 826 in the second reading frame, which potentially encodes a protein of M_r 27,000 and is consistent with the reading frame used by SFV RNA. The product of approximately M_r 25,000 from pSFVDI-19 RNA may have been derived from the ORF that extends from nucleotides 86 to 667 in the second reading frame, or from the ORF that extends from 549 to 1055 in the third. However, since the estimated size of proteins from these two ORFs is only 21,000 and 19,000 respectively, it is not clear how this protein was produced

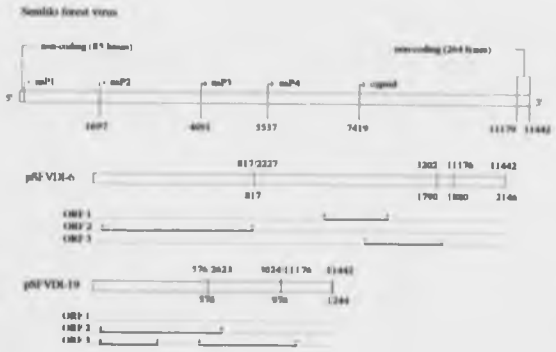


Figure 6.4 Open reading frames of pSFVDI-6 and pSFVDI-19

The diagram of the SFV genome shows the non-coding and coding regions. Non-structural proteins (nsP1-nsP4) are translated in the second reading frame, and the capsid proteins in the third. The diagrams of pSFVDI-6 and pSFVDI-19 show the nucleotides at the junctions of homology with SFV RNA above the sequences, and the equivalent nucleotides of the clones below. Open reading frames of greater than 200 nucleotides in length are shown below the sequences (labelled ORF1-3)

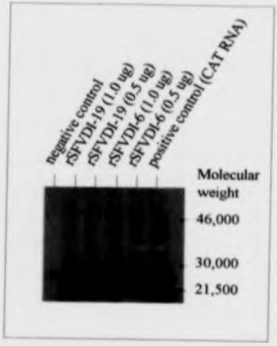


Figure 6.5 *In vitro* translation products of pSFVDI-6 and pSFVDI-19 RNA transcripts

0.5 or 1.0 µg of RNA was used in a rabbit reticulocyte lysate translation system with [^{35}S]-methionine as label. Translation products were analysed by SDS-PAGE on a 10-20% gradient gel. The positions of unlabelled molecular weight markers are indicated on the right. The positive control mRNA was chloramphenicol acetyl transferase (CAT), which gives a translation product of 24,000. The main translation products of pSFVDI-19 and pSFVDI-6 RNA transcripts had a M_r respectively of approximately 25,000 and 27,000 (arrowed).

DISCUSSION

RNA transcripts were shown to be of the same size as their respective DNA templates and they were produced in sufficient quantities for subsequent transfection experiments. It was assumed from the high fidelity of T3 RNA polymerase that these transcripts were copied accurately from their corresponding DNAs and that it was unnecessary to investigate them further.

One concern was that the additional 5'-guanine residue derived from the promoter that was used would be detrimental to the subsequent propagation of the RNA transcripts as virions. In preliminary experiments not described here, a T3 RNA polymerase promoter was used which required the same residue at the transcription start site as the 5' terminal nucleotide of the DI SFV genomes. RNA transcribed using this promoter could not be detected by standard methods, suggesting that the promoter was too weak to enable sufficient quantities of RNA to be produced for subsequent transfection. It was decided to compromise by using a promoter that was powerful but required a different residue at the transcription start site from the 5' terminal nucleotide of the cloned DI SFV genomes. Liljeström *et al.* (1991) found that RNA transcribed from the SFV clone pSP6-SFV4, which also included a guanine residue at the 5' terminus, had an infectivity titre in tissue culture comparable to that of virion RNA. It was likely, therefore, that propagation of the virus was not altered significantly by this extra nucleotide, suggesting that propagation of defective RNAs, which was likely to involve the same mechanisms as those for standard virus, would also proceed satisfactorily.

It is possible that non-viral nucleotides were incorporated at the 3' termini of the transcribed RNAs. Because the substrate for RNA polymerases is double-stranded DNA, it was assumed that when the enzyme reached the cleavage site created by the restriction enzyme it would not continue to transcribe from the non-viral nucleotides comprising the single-stranded portion of the digested site. It was found subsequently that RNA transcripts produced from pSFVDI-6 could be propagated as virions, hence putative additional nucleotides were not inhibitory to this process (see Chapter 7). It was therefore not considered necessary to investigate this further because all the DNA templates that were used, apart from pSFVDI-SB1, possessed the same transcriptional features as those of pSFVDI-6.

Amplification of DI SFV-p7 RNAs by RT-PCR has already been discussed (Chapter 3). There was concern that the use of primers with additional non-SFV sequences at the 3' termini would prevent the amplification of DI SFV genomes. However, RNA species detected using primers with transcriptional features were, with respect to size, the same as those detected using *5'SP6'* and *3'SP6'* RNA transcribed from these PCR products comprised just two predominant species of 2.1 and 1.2 kb. It is likely that the process of RT-PCR using DI SFV-p7 selected particular DI genomes, and that these were further selected for in the process of RNA transcription. Hence a limited range of putative DI SFV genomes was defined. As stated previously, these were screened for mouse-protecting activity to determine if they should be cloned.

It is possible that errors could be introduced into the nucleotide sequence by amplifying from pSFVDI-1A2 using PCR in order to provide a corrected template for RNA transcription. It is also possible that through non-specific hybridisation of

the PCR primers to pSFVDI-1A2 other species of DNA would be produced and hence subsequent transcription would yield a heterogeneous preparation of RNA. Minor DNA species were not detected in the PCR product derived from pSFVDI-1A2, but it is not known how accurately the SFV-derived sequences of the clone had been copied. In retrospect, it may have been advantageous to reconstruct pSFVDI-1A2 by deletion of the modified form of pSFVDI-6.

While both rSFVDI-6 and rSFVDI-19 encoded proteins in an artificial translation system, it is not known if such proteins are produced in tissue culture or *in vivo*, or if these proteins contribute in any way to interference or protection. Previously cloned DI SFV genomes do not have extensive open reading frames (ORF) (Lehtovaara *et al.*, 1981, 1982). It is probable, therefore, that the production of proteins from DI SFV genomes is not fundamental to the biological activity of all DI SFV. Some DI genomes of other viruses, for example Ross River virus and influenza virus, encode truncated versions of standard virus proteins (Martin *et al.*, 1979; Penn and Mahy, 1985, reviewed in Nayak *et al.*, 1989). The contribution, if any, of these proteins to the biological activity of the DI genomes is not known.

rSFVDI-6 encoded a protein of M_r 27,000, which was assigned to an ORF on the basis of the predicted coding potential of this region of sequence. Further experimentation is needed to confirm the origin and nature of this protein. The protein of M_r 25,000 encoded by rSFVDI-19 could not be assigned to any particular ORF. It is not possible, without further investigation, to resolve the discrepancy between the size of the protein produced and the potential of rSFVDI-19 to encode such a protein. It is possible that the protein had an electrophoretic mobility that was reduced in relation to the proteins comprising the size marker.

CHAPTER 7

TRANSFECTION AND RESCUE OF CLONED DEFECTIVE RNAs OF SEMLIKI FOREST VIRUS

INTRODUCTION

This chapter describes the transfection of RNA transcripts produced from clones of defective SFV into tissue culture cells and the detection of DI virus derived from these transcripts.

While infection of eukaryotic cells by SFV is mediated via receptors for virion proteins, there are no known natural receptors for SFV RNA. To investigate the biological activity of cloned defective genomes it is therefore necessary to produce virus particles by encapsidating the nucleic acid. This can be achieved by introducing the RNA into cells harbouring infectious virus which thus provide the proteins required for replication and encapsidation. In order to introduce RNA into such cells, the process of transfection can be used. In this, cells are stimulated by chemical or mechanical means to take up foreign nucleic acid.

A number of methods have been used previously for the transfection of RNA into tissue culture cells. These include hypertonic shock and using DEAE-dextran and more recently, lipofection and electroporation.

To transfect RNAs derived from clones of DI Sindbis virus into tissue culture cells, Levis *et al* (1986) used the method based on DEAE-dextran. This method would probably have been applicable to the work described here but Liljeström *et al* (1991) reported 100% transfection of RNA transcripts from the full-length SFV clone pSP6-SFV4 by electroporation. They also showed that the equivalent efficiency using the DEAE-dextran method was only 0.2% under optimal conditions. Electroporation was therefore used in transfection experiments.

although attempts were made initially to transfect RNA using the method of hypertonic shock. This chapter describes the adaptation of the method of electroporation to the transfection of RNA transcripts from defective SFV clones. The success of these experiments was determined by analysis of tissue culture fluid. Because RT-PCR had been used successfully to analyse heterogeneous preparations of DI SFV, the same method was used to detect putative DI virus derived from transfected RNA. In addition, tissue culture fluid was analysed for interference in BHK-21 cells because such activity would suggest that virions were being formed and released and that they contained DI genomes. An analysis *in vivo* for mouse-protecting activity of these preparations is described in the following chapter.

To determine the relative concentrations of DI SFV in preparations derived from cloned genomes, attempts were made to quantitate SFV-derived RNA extracted from tissue culture fluid. This chapter describes the adaptation of dot blot analysis for this purpose, and discusses the limitations of the technique for quantitating DI SFV RNA.

This chapter also describes the propagation of deletion mutants of pSFVDI-6 as virus particles. In addition, the propagation of RNA transcripts produced from the artificially constructed defective SFV clone, pSFVDI-SB1, is described. The purpose of these experiments was to determine if the regions of pSFVDI-6 derived from the nsP2 gene of SFV were necessary for propagation as virions and to permit speculation on the nucleotide sequences required for the propagation of DI SFV genomes.

RESULTS

1. Transfection of RNA transcripts into BHK-21 cells

As stated in Chapter 6, RNA transcripts derived from clones or PCR products have been given the prefix 'r' (e.g. 'rSFVDI-19'). For clarity, preparations of virus derived from transfection experiments have been given the prefix 'v' (e.g. 'vSFVDI-19'). The passage number of such virus preparations is indicated after the name (e.g. vSFVDI-19/p3). virus in tissue culture fluid harvested following transfection is designated passage 1.

(a) Methodology

The success of transfection was initially determined by using RT-PCR with the primers *5'SP1'* and *3'SP1'* to detect RNAs in culture medium following transfection. Such RNA was assumed to have been encapsidated into virions. The *in vitro* interference activity of any DI SFV present in culture medium was also assayed (see section 3a).

In the first experiments, attempts were made to transfect RNA into BHK-21 cells using hypertonic shock. Following transfection of RNA extracted from a tissue culture preparation of SFV, infectious virus was detected in the tissue culture fluid, suggesting that this method was successful. However, after transfection of RNA derived from the defective SFV clones using SFV for propagation, DI SFV was not detected in tissue culture fluid harvested from the first and subsequent passages by RT-PCR or by interference assay.

In later experiments, the electroporation procedure described by Liljeström *et al* (1991) was adapted for the transfection of RNA transcripts derived from clones of defective SFV. Monolayers of BHK-21 cells were infected 45 minutes before transfection with 10 p f u /cell SFV. In a typical experiment approximately 20µg RNA were used, which equates to 2 x 10^6 copies per cell, assuming a genome size of 2.0 kb. After electroporation, cells were incubated for 18-24 hours and tissue culture fluid harvested. To amplify putative DI SFV, culture medium was serially passaged in BHK-21 cells at least 3 times, with 10 p f u /cell SFV added each time.

The electroporation procedure was assessed for lethal effects upon cells. In preliminary experiments, the cells that did not attach to the tissue culture flask were counted 2 hr after electroporation. These comprised less than 1% of the total. Assuming that all attached cells were viable, the electroporation procedure was not significantly deleterious to cell viability.

In preliminary experiments the efficiency of transfection was determined. Following electroporation with RNA transcripts derived from pSP6-SFV4, an infectious centre assay was used to measure the proportion of cells harbouring infectious RNA. In two such experiments the average efficiency was 30%. While this was significantly lower than the transfection efficiency achieved by Liljeström *et al.* (1991), it was considered sufficiently high for the purposes of establishing homogeneous DI virus populations from defective RNA transcripts following transfection.

(b) Control samples

False positive results were frequently obtained when RT-PCR was used to detect DI SFV in culture medium following transfection. It was likely that this was a consequence of plasmid DNA contaminating the inoculum. RNA transcripts were routinely treated with DNAse I before transfection to remove template DNA, but this may not have been completely effective. It was therefore necessary to routinely include appropriate control samples. Non-infected cells, transfected with 20μg of the RNA under test, were used as a negative control. A virus-alone control was also included to permit analysis of the generation of DI SFV *de novo*. To determine that PCR products were derived from RNA rather than DNA, RT-PCR was performed without reverse transcriptase. Finally, a positive control for the transfection procedure was included, in which SFV-infected cells were transfected with RNA transcripts that had been shown in other experiments to be propagated as virus particles. In the first experiments, RNA derived from pSP6-SFV4 was used to determine the success of the procedure.

(c) Virus stocks

To create cloned stocks of DI virus, it was desirable to use infectious helper virus that was free of DI virus. As DI SFV was not propagated or generated in HeLa cells (N.J. Dimmock, unpublished data) a HeLa-grown stock of SFV was used initially. However, RT-PCR analysis of culture medium after transfection of rSFVDI-6 using this virus stock suggested that this RNA was not propagated as virus particles (data not shown). In subsequent experiments, an SFV stock was produced by transfecting rSP6-SFV4 into BHK-21 cells, followed by a single low-

multiplicity passage. This preparation was designated vSFV4-p2/F13. Transfection of defective RNA transcripts using vSFV4-p2/F13 as the source of helper virus was successful as determined by RT-PCR analysis of tissue culture fluid. All transfection experiments described below used this stock of SFV. It was found subsequently that propagation of defective RNA transcripts as virus particles was unsuccessful using another stock of SFV produced in the same way as vSFV4-p2/F13.

PCR products derived from transfection experiments using vSFV4-p2/F13 typically contained spurious DNA species of 0.6 and 1.1 kbp. DI SFV was not detected in this virus stock by an interference assay (see Table 7.1). In addition, RNA species of these sizes were not detected by northern blot analysis (see below). These results suggested that these PCR products resulted from the PCR primers hybridising to virion RNA non-specifically (this has already been discussed, see Chapter 3).

(d) Transfection of rDI7

RNA transcripts were produced from PCR products derived from the mouse-protecting tissue culture preparation DI SFV-p7 (rDI7, see Chapter 6). These were transfected into BHK-21 cells and the tissue culture fluid serially passaged 5 times. Analysis by RT-PCR showed that after passages 1 and 2, a DI SFV genome of 1.2 kb was present in the tissue culture fluid (Figure 7.1). PCR products derived from later passages were heterogeneous and the 1.2 kbp species no longer predominated. The 1.2 kbp product was shown by restriction analysis as similar in structure to the SFV-derived sequences of pSFVDI-19 (data not shown). Figure

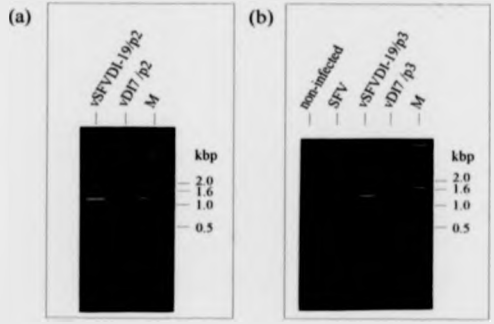


Figure 7.1 Transfection of rDI7 into SFV-infected BHK-21 cells

RNA was detected in culture medium by RT-PCR with the primers 5'NT (
3'NT. In **(a)**, the predominant PCR product from vDI7/p2 is 1.2 kbp (arrowed), the same size as that from vSFVDI-19/p2, but in lower quantities. In **(b)**, several other products appear on amplification of RNA extracted from vDI7/p3 in contrast to that from vSFVDI-19/p3. The band of 0.6 kbp that appears in the amplification products of all samples is explained in the text.

7.1 also shows PCR products derived from subsequent RT-PCR analysis of vSFVDI-19. In contrast to the PCR products derived from vDI7, these products did not vary significantly with passage number.

(e) Transfection of rSFVDI-6

rSFVDI-6 was transfected into SFV-infected BHK-21 cells and culture medium serially passaged 9 times in BHK-21 cells. Figure 7.2 shows PCR products derived from passage 3. A predominant species of 2.1 kbp was detected only in the PCR product derived from infected cells transfected with rSFVDI-6. This was the same size as the product obtained after amplification from pSFVDI-6 using the same primers. These results suggested that the RNA had been successfully rescued as virus particles. The origin of the 1.1 kbp DNA produced by RT-PCR using the vSFVDI-6 and virus-alone samples is discussed above.

In a restriction analysis of the PCR product derived from vSFVDI-6/p3, the sizes of the fragments obtained after digestion with HaeIII corresponded to those predicted from the nucleotide sequence of pSFVDI-6. This and other restriction analyses suggested that the genome structure of pSFVDI-6 did not alter significantly in the processes of transcription, transfection and serial passage.

Figure 7.3 shows PCR products derived from passages 1 to 9 of vSFVDI-6. A product of 2.1 kbp was detected in all samples. Analysis of these products by digestion with HaeIII suggested that all contained sequences derived from pSFVDI-6 (data not shown).

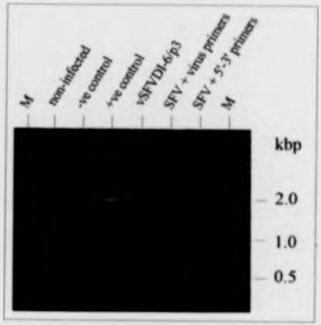


Figure 7.2 Transfection of rSFVDI-6 into SFV-infected BHK-21 cells

(a) RNA in culture medium was detected by RT-PCR with the primers *5'SFV* + *3'SFV*. As a negative control, RT-PCR was performed without reverse transcriptase. As a positive control for PCR, pSFVDI-6 was amplified with the primers *5'SFV* + *3'SFV*. The predominant product of 2.1 kbp derived from vSFVDI-6 matched the size of the product from the positive control. Amplification of RNA derived from the virus-alone controls yielded a product of 0.7 kbp using the primers *SFV-5i* and *SFV-3i* and one of 1.1 kbp using the primers *5'SFV* and *3'SFV*. The latter matched the size of a minor product obtained after amplification of RNA derived from vSFVDI-6/p3.

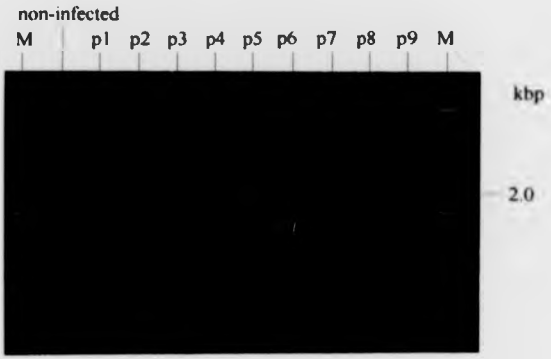


Figure 7.3 Detection of defective SFV RNA in passages 1 to 9 of vSFVDI-6

RNA was extracted from tissue culture fluid, reverse transcribed and amplified by PCR using the primers *5'SFV* + *3'SFV*. DNA of 2.1 kbp was detected in the PCR products derived from passages (p) 1 to 9 of vSFVDI-6

(f) Transfection of rSFVDI-19

Figure 7.4 shows PCR products derived from tissue culture fluid harvested 3 passages after transfection of rSFVDI-19 into SFV-infected BHK-21 cells. A 1.2 kbp product was obtained from the vSFVDI-19 sample, which corresponded to the size of the SFV-derived sequences of pSFVDI-19. Figure 7.5a shows PCR products derived from passages 1 to 9 of vSFVDI-19. In all these, a 1.2 kbp product was detected. Digestion of these products with AccI produced fragments corresponding to those predicted from the restriction map of pSFVDI-19 (Figure 7.5b).

(g) Analysis of vSFVDI-6 and vSFVDI-19 by northern blotting

Putative DI SFV preparations derived from pSFVDI-6 and pSFVDI-19 were analysed by northern blotting to determine if they contained RNA species undetected by RT-PCR. RNA extracted from passage 3 was resolved on an agarose gel after denaturation with glyoxal. Following transfer to a nylon filter, RNA was hybridised to an RNA probe. This probe was made by transcribing EcoRI-digested pSFVDI-7 (see Chapter 4) with T7 RNA polymerase to generate negative-sense RNAs, which were labelled with [α-^{32}P]UTP. A 2.1 kb species was detected in vSFVDI-6/p3 and three species of 1.2, 2.4 and 3.6 kb were detected in RNA derived from vSFVDI-19/p3 (Figure 7.6). It is likely that the RNA derived from vSFVDI-19/p3 had not denatured sufficiently because the sizes of the larger RNA species were multiples of the size of the cloned genome, suggesting the molecule had concatamerised. No RNAs other than these were detected.

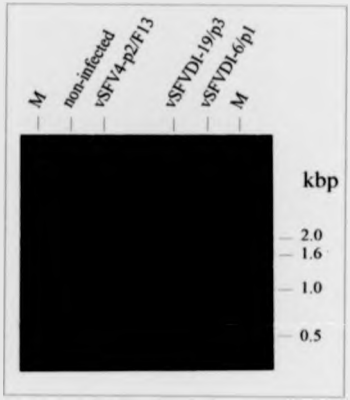


Figure 7.4 Transfection of rSFVDI-19 into SFV-infected BHK-21 cells

RNA was detected in culture medium by RT-PCR with the primers *5'SFV* + *3'SFV*. The PCR products from vSFVDI-19/p3 and vSFVDI-6/p1, of 1.2 and 2.1 kbp respectively, corresponded to the sizes of the two cloned molecules

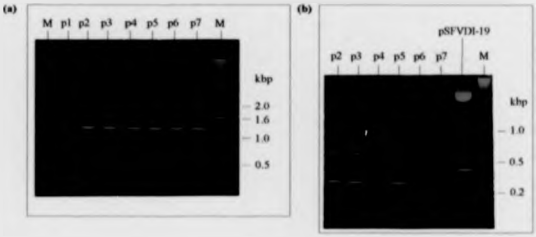


Figure 7.5 Detection of defective SFV RNA in passages 1 to 7 of vSFVDI-19

(a) RNA was extracted from tissue culture fluid, reverse transcribed and amplified by PCR using the primers *5'SFV* + *3'SFV*. DNA of 1.2 kbp was detected in the PCR products derived from passages (p) 1 to 7 of vSFVDI-19.

(b) PCR products derived from passages 2 to 7 of vSFVDI-19 were digested with AccI. The band patterns produced after agarose gel electrophoresis were the same in all samples. Digestion of pSFVDI-19 with AccI produced a similar pattern of bands: the additional 3.0 kbp band comprised vector sequences and included 0.3 kbp that derived from the 3' terminus of the clone.

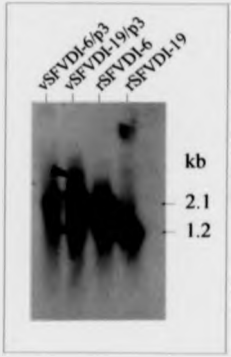


Figure 7.6 Analysis of vSFVDI-6 and vSFVDI-19 by northern blotting

RNA extracted from vSFVDI-6/p3 and vSFVDI-19/p3 was resolved on an agarose gel after denaturation with glyoxal. Following northern transfer, RNA was hybridised to a [α-^{32}P]UTP-labelled RNA probe (rSFVDI-7). Comparison to rSFVDI-6 and rSFVDI-19 and to unlabelled size markers showed that a single RNA species of 2.1 kb was present in vSFVDI-6, and RNAs of 1.2, 2.4 and 3.6 kb were present in vSFVDI-19 (arrowed).

suggesting that the defective SFV preparations were homogeneous In addition, these results suggested that the 0 6 and 1 1 kbp PCR products derived from RT-PCR amplification of SFV RNA (see above) did not comprise defective SFV genomes

2. Assessment of the propagation as virions of deletion mutants of pSFVDI-6 and the artificially constructed clone pSFVDI-SB1

Chapter 4 described the construction of various clones by centrally deleting pSFVDI-6 pSFVDI-1A2 was constructed by deleting the region of pSFVDI-6 from AccI (534) to AccI (1453), and pSFVDI-M2 was made by deleting the region of pSFVDI-6 from MluI (1225) to MluI (1690) pSFVDI-SB1 was made by deleting the full-length SFV clone, pSP6-SFV4, from SalI (1658) to BstEII (10920) To determine if these clones retained all the sequences necessary for propagation as virions they were transfected into SFV-infected BHK-21 cells and tissue culture fluid was subsequently analysed by RT-PCR and interference assay

(a) Transfection of rSFVDI-SB1

RNA transcripts produced from pSFVDI-SB1 were transfected by electroporation into SFV-infected BHK-21 cells After 3 passages tissue culture fluid was analysed by RT-PCR (Figure 7 7) While the positive control sample showed that the transfection was successful, RNA corresponding to the size of the transfected RNA (2 1 kb) was not detected, suggesting that rSFVDI-SB1 had not been propagated as virus particles

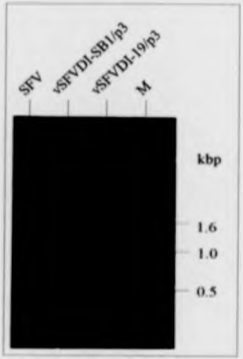


Figure 7.7 Transfection of rSFVDI-SB1

rSFVDI-SB1 was transfected into SFV-infected BHK-21 cells. After 3 passages, RT-PCR with the primers *5'NA1'* and *3'NA1'* was used to detect putative DI SFV in tissue culture fluid. The positive control was rSFVDI-19. RNA of 1.2 kbp was detected in vSFVDI-19. No RNA that corresponded to the size of the SFV-derived sequences of pSFVDI-SB1 (2.1 kbp) was detected in vSFVDI-SB1.

(b) Transfection of rSFVDI-1A2 and rSFVDI-M2

Figure 7.8 shows PCR products derived from vSFVDI-1A2 and vSFVDI-M2. The positive control sample (vSFVDI-6) showed that the transfection was successful. RNA corresponding to the size of rSFVDI-M2 was not detected in tissue culture fluid. An RNA genome of 1.1 kb was detected in tissue culture fluid derived from transfection with rSFVDI-1A2. However, this was approximately 0.2 kb less than the size of the SFV-derived sequences of pSFVDI-1A2. Further investigation is needed to determine the nature of this PCR product and to prove whether or not rSFVDI-1A2 was successfully propagated as virus particles.

3. Qualitative and quantitative assessment of DI SFV preparations

(a) Assessment of biological activity of DI SFV preparations *in vitro*

Table 7.1 shows the relative tissue culture (BHK-21 cell) interference titres of the various DI SFV preparations. Note that the values shown do not take account of the quantity of DI particles in a given population. The estimations of the relative concentrations of DI SFV in the various preparations were made by visual examination of the PCR product on an agarose gel. These estimations were made assuming that RT-PCR amplified different RNA species with equal efficiency.

It is clear from these results and from quantitation of total viral RNA by dot hybridisation (see below) that vSFVDI-6 was not propagated as efficiently as vSFVDI-19. The large quantities of PCR product from amplification of vSFVDI-1A2 RNA suggested abundance of an RNA species derived from rSFVDI-1A2 in

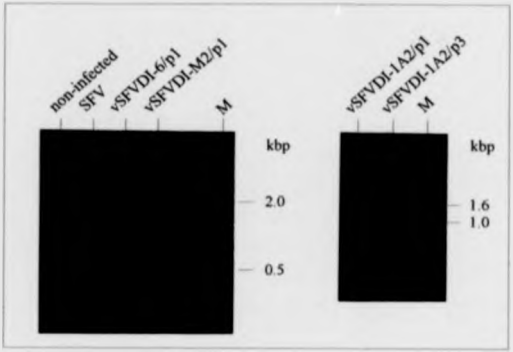


Figure 7.8 Transfection of rSFVDI-M2 and rSFVDI-1A2

Following transfection of rSFVDI-M2 and rSFVDI-1A2 into SFV-infected BHK-21 cells, RT-PCR was used to detect putative DI SFV genomes in tissue culture fluid. rSFVDI-6 was included in the experiment as a positive control. No DNA corresponding to the size of pSFVDI-M2 (1.7 kbp) was detected in the PCR product derived from vSFVDI-M2. DNA of 1.1 kbp was detected in PCR products derived from vSFVDI-1A2/p1 and p3. A 0.6 kbp PCR product was detected in all samples derived from SFV-infected cells.

Table 7.1 *Interference titres of DI SFV preparations.* *

SFV DI preparation †	DI genome size (nucleotides)	Passage number	Relative concentration ‡	1/highest dilution showing no cytopathic effect §
DI SFV-p7	NA	7	+	128
vSFVDI-6	2146	3	++	16
vSFVDI-19	1244	3	+++	64
vSFVDI-1A2	1228	5	++	2
vDI7	NA	2	+	4
virus (p3)	NA	3	-	2
virus (p9)	NA	7	+++	64

* BHK-21 cells were used, at a concentration of 2×10^4 cells per well

† Each of the cloned DI preparations was u v -irradiated for 80 seconds to remove infectivity. No cytopathic effect (c p e) was observed in wells containing DI SFV alone. vSFV4-p2/F13, used in the propagation of the cloned DI RNAs, was included as a negative control

‡ These were estimated by visual examination of the PCR product on an agarose gel

§ Each DI virus preparation was assayed in quadruplicate by serial two-fold dilution in medium containing 10 $TCID_{50}$ SFV. c p e was assessed 24 hours after infection

NA = not applicable

the cell culture medium As stated previously, it was not proven that rSFVDI-1A2 had been propagated as virus particles This population did not interfere significantly, suggesting either that rSFVDI-1A2 had not been propagated or that some property of pSFVDI-6 responsible for interference in cell culture was lost in the creation of this mutant Table 7 1 also shows that vDI7 interfered weakly in cell culture In addition, low levels of PCR product from amplification of vDI7 RNA suggested that this population was propagated poorly

As stated previously, the generation of DI SFV *de novo* was assessed by passaging the virus used for transfection (vSFV4-p2/F13) in parallel with the test samples Table 7 1 shows that after 3 passages of virus-alone the interfering activity was negligible, but this increased to levels similar to that of vSFVDI-19 after 9 passages A 0 8 kb RNA in the tissue culture fluid of passage 9 was suspected to be responsible for this interfering activity (see Chapter 3)

(b) Quantitation of vSFVDI-6 and vSFVDI-19 by dot blot analysis

To investigate the proportion of the total virus population that DI SFV comprised in vSFVDI-6 and vSFVDI-19, attempts were made to measure relative quantities of SFV-derived RNA RT-PCR was used initially for this, but the results were ambiguous and were not reproducible Subsequently, total SFV-derived RNA was measured by dot blot analysis

vSFVDI-6/p3 and vSFVDI-19/p3 were prepared in the same experiment These preparations and vSFV4-p2/F13 were diluted to the same infectivity titres (10^8 p f u /ml), as determined by plaque assay, and RNA was extracted from the diluted

tissue culture fluid. Figure 7.9 shows RNA samples analysed in quadruplicate by dot blotting using an [α-^{32}P]-labelled RNA probe (rSFVDI-7, see above). Relative quantities of hybridised RNA were determined using a scanning densitometer (Table 7.2). The value obtained for the virus-alone sample was subtracted from the test samples, giving values assumed to correspond to RNA derived from DI virus. Estimates of the proportions of DI SFV that comprised vSFVDI-6 and vSFVDI-19 were made by comparison of these values with that obtained for virus alone. Table 7.2 shows that the relative proportions of DI SFV RNA derived from vSFVDI-6 and vSFVDI-19 was 1:15.

For this analysis, the probe was assumed to hybridise to all SFV-derived RNAs with equal efficiency. In addition, the density values of each blot were assumed to be directly proportional to the number of SFV-derived RNA molecules. However, it was known from the analysis shown in Table 7.2 and from repeated experiments that the difference in density values between dilutions of the same sample were not necessarily proportional to the dilution factor. Because the exposure curve of X-ray film is non-linear, it was likely that measurements made at both the lower and upper ranges of optical density were not directly proportional to the degree of hybridisation. For these reasons, intermediate density values were assumed to be the most precise.

(c) Nucleotide sequences required by rSFVDI-6 for propagation as virus particles.

To determine the regions of DI SFV genomes required for propagation, the nucleotide sequences of the defective SFV clones used in the transfection

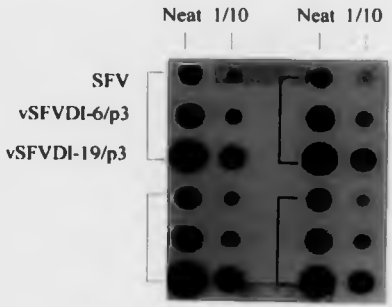


Figure 7.9 Analysis of vSFVDI-6/p3 and vSFVDI-19/p3 by dot blotting

Equal quantities of rSFVDI-6 and rSFVDI-19 were transfected into SFV-infected BHK-21 cells. After 3 passages, tissue culture samples vSFVDI-6, vSFVDI-19 and the virus-alone control were diluted to 10^8 p.f.u./ml and RNA was extracted from the dilutions. RNA was transferred to a nylon filter and hybridised with an [α-^{32}P]UTP-labelled RNA probe (rSFVDI-7). Samples were analysed in quadruplicate (denoted by brackets), either undiluted or at a dilution of 1 in 10. The relative densities of each blot were determined using a scanning densitometer (see Table 7.2).

Table 7.2 *Quantitation of SFV-derived RNAs by densitometry* *

Virus preparation	Dilution	Integrated volume †	Volume attributable to DI SFV RNA ‡	Ratio of SFV RNA to DI SFV RNA §
vSFV4-p2/F13	Neat	281	0	1 0
	1/10	24	0	
vSFVDI-6	Neat	611	330	1 1
	1/10	66	42	
vSFVDI-19	Neat	2187	1906	
	1/10	448	424	1 15

* The RNA blot shown in Figure 7 9 was used in this analysis

† The integrated optical density of each dot blot was determined by computer analysis of the scanned X-ray film Numbers are in arbitrary units

‡ The density values determined for the virus-alone samples were subtracted from those of the DI SFV samples The virus-alone sample was assumed to consist solely of SFV RNA for this calculation

§ Only intermediate values were used because they were suspected to be more precise, for reasons discussed in the text Ratios were determined by division of the values representing DI SFV RNA with that for virus alone

experiments were compared. Table 7.3 summarises the regions of the clones that were derived from the nsP2 gene of SFV and shows that part of this gene is essential for propagation of virus particles. pSFVDI-SB1 has none of the nsP2 sequence present in pSFVDI-6 and was not propagated as virions. In addition, the non-propagated pSFVDI-M2 lacked some of the sequences of pSFVDI-6 that were derived from the nsP2 gene. By subtraction, the sequence required for propagation lies with the 466 nucleotides from 1225 to 1690 of pSFVDI-6, these equate to 2637 to 3102 of the nsP2 gene of the virion. If pSFVDI-19, which has nucleotides 2623 to 3024 of nsP2, is considered in addition, it is possible to define a smaller region of 388 nucleotides, from 2637 to 3024 of SFV RNA, that are needed for propagation of virions. Table 7.3 also shows that this region is conserved in DI 309 (Lehtovaara *et al*., 1982), further supporting the above conclusions.

Table 7.3 *Analysis of the sequence requirement for propagation of DI genomes as virus particles.*

Clone	pSFVDI-6	pSFVDI-M2	pSFVDI-SB1	pSFVDI-19	DI 309 ‡
propagation	+	-	-	+	+
sequence derived from nsP2 gene*	2227-3202	2227-2636 3103-3202	none	2623-3024	2627-3063
sequence absent from pSFVDI-6†	NA	2637-3102	2227-3202	2227-2622 3025-3202	2227-2626 3064-3202

* Numbers refer to nucleotide positions in SFV RNA

† Only pSFVDI-6 sequences derived from the nsP2 coding region of SFV RNA are considered here

‡ Lehtovaara *et al.*, 1982

NA = not applicable

DISCUSSION

Transfection of defective RNA transcripts into SFV-infected BHK-21 cells by electroporation generated detectable quantities of defective SFV RNA after a single passage. RNA transfection by hypertonic shock gave no detectable defective RNA, suggesting that the proportion of cells receiving RNA was not sufficiently high to establish a homogeneous DI SFV population. Levis *et al.* (1986) showed that following transfection of certain defective Sindbis RNA transcripts using the DEAE-dextran method, several defective RNA species were detected in the culture medium in addition to the RNA of interest. It is possible that DI genomes generated *de novo* are not competed out by low amounts of input RNA transfected at low efficiency.

Interfering activity was detected in vSFVDI-6 and vSFVDI-19 after 2 passages. In contrast, this activity was detected in the virus control samples after 4 to 6 passages. In addition, these preparations contained only the DI RNA that they were expected to have. It is reasonable to conclude, therefore, that the defective genomes within pSFVDI-6 and pSFVDI-19 possessed sequences required for interference *in vitro* and they could therefore be defined as defective interfering genomes. Evidence from RT-PCR and northern blot analyses (discussed below) suggested that the cloned DI SFV preparations were homogeneous. It is therefore likely that the interfering activity of these preparations was attributable to the interfering sequences derived from the cloned genomes. From this premise, the biological activities of the cloned DI SFV preparations *in vitro* and *in vivo* can be defined on the basis of their constituent DI sequences.

Propagation of defective RNA transcripts as virions was observed after transfection using vSFV4-p2/F13 as helper, but not after transfection using either HeLa-grown virus or another preparation of SFV derived from pSP6-SFV4. There is clearly some function of the SFV genome relevant to the propagation of these DI clones, which varies between virus preparations. This function may be analogous to the altered replicase specificity described for a mutant of VSV that was resistant to DI particles (Sdi mutant: Giachetti and Holland, 1988). However, after several passages of each the virus preparations, DI SFV was detected by interference assay, showing that these preparations comprised virus that was not resistant to interference by DI virus. A preliminary experiment in which an established preparation of vSFVDI-6 was passaged using HeLa-grown virus suggested that replication and amplification of the genome was unaffected but the abundance of DI RNA derived from vSFVDI-6 was reduced (data not shown). This suggests that the difference in these virus preparations relates to the encapsidative rather than the replicative functions. It is possible that the non-propagation of cloned DI SFV genomes *in vitro* using certain virus preparations is analogous to the *in vivo* system. As will be discussed in the following chapter, the degree of mouse-protection by co-administration of SFV and DI SFV is dependent on the preparation of SFV used.

Once cloned DI SFV populations were established, they could be maintained through at least 9 passages, showing that they were stable and suggesting that they prevented the establishment of DI genomes *de novo*. This dominance effect has also been seen with DI VSV and DI influenza virus (VSV: reviewed in Holland, 1985, influenza virus: reviewed in Nayak *et al*., 1989). Restriction analysis of the PCR products derived from different passages suggested that the overall structure

of the cloned defective genomes did not alter during their propagation *in vitro*. The stability of cloned defective RNA contrasts with the instability on passage of the heterogeneous mouse-protecting population DI SFV-p7 (see Chapter 3). The PCR profile derived from DI SFV-p7 changed after a single passage, from exhibiting two main products of 2.1 and 1.2 kbp to showing only the 2.1 kbp product. The artificial enrichment of the 1.2 kb species through transcription of the PCR product and subsequent transfection permitted maintenance through two passages (vDI7/p1 and p2), but it was again lost after the third. This could be explained by the competing action of minor species of defective RNAs present in the transcription products, and underlines the necessity of homogeneous transcripts to generate homogeneous RNA populations.

Although RT-PCR analysis suggested that the cloned DI genomes were successfully propagated as virions, it is possible that other DI species were present but were not detected because of the limitations of the technique (discussed in Chapter 3). The analysis of vSFVDI-6 using northern transfer suggested that other SFV-derived sequences were not present. Analysis of vSFVDI-19 revealed RNAs that were multiples of the size of the cloned genome, suggesting the molecule had concatamerised. As was discussed in Chapter 5, SFV RNA can circularise because of complementary sequences at the termini. Because the cloned DI SFV genomes possess both termini of the standard virus genome, it is conceivable that concatamerisation could occur if the RNA is in a sufficiently high concentration. Analysis of the relative quantities of vSFVDI-6 and vSFVDI-19 by dot blotting indicated that the latter was approximately 15 times as abundant. Because the same volumes of each preparation were used in the northern analysis, more vSFVDI-19 RNA was used in the analysis than RNA from vSFVDI-6, and it was therefore

more likely to be incompletely denatured However, further investigation is needed to confirm these conclusions

The estimations of the relative quantities of vSFVDI-6 and vSFVDI-19 suggested that the latter was propagated *in vitro* more efficiently It is possible that the smaller DI genome of pSFVDI-19 was replicated more frequently within the infected cell and as a consequence the DI virus population became established more rapidly However, because the dot blot analysis measured relative amounts of SFV-derived RNAs and not the absolute numbers of DI virus particles, it is not possible to make firm conclusions on the relative efficiencies with which the two DI genomes were propagated as virions However, evidence from the u v -target size of DI SFV suggested that one genome was packaged per virion (Barrett *et al* , 1981) Whether this is also true of vSFVDI-19, which consists of a genome that is approximately half the size of previously studied DI SFV genomes, is not known, but there are no reasons to suspect that multiple copies of this genome would be packaged into a single virion Bruton and Kennedy (1976) showed that the buoyant density of DI SFV on a CsCl velocity gradient was greater than that of standard SFV They concluded that this was either a consequence of each virus particle packaging multiple copies of the DI genome or the DI SFV particles possessed more compact nucleocapsids The latter conclusion is supported by evidence from electron microscopy that DI SFV particles are smaller than those of standard virus (Barrett *et al* , 1984b) It is conceivable, therefore, that particles which package single copies of DI SFV genomes can be denser than standard virus particles, despite having less RNA

The methodology used to establish homogeneous populations of DI SFV proved a rapid and effective means of determining if artificially generated DI genomes could be propagated as virus particles. Much has still to be done to determine the functions of the various regions of the cloned DI SFV genomes, but this aim could readily be achieved with the methodology described in this thesis. A sequence of 388 nucleotides, corresponding to 2637 to 3024 within nsP2 of the virion was defined, which was necessary for the propagation of DI virions. This could contain a packaging signal analogous to that identified within nsP1 of Sindbis virus or within the polymerase gene of the coronavirus MHV-4, another positive-strand RNA virus (Levis *et al*., 1986, Makino *et al*., 1990). Following transfection of rSFVDI-1A2, an RNA species was detected by RT-PCR in culture medium that was approximately 0.2 kb smaller than expected. Comparison of the PCR profile obtained from vSFVDI-1A2 with those derived from the control samples suggested that this RNA had derived from the transfected genome. It is possible that the genome altered during its amplification and propagation *in vero*, for example through recombinational events. Interfering activity was not detected in vSFVDI-1A2, suggesting that the RNA that was propagated as virions did not possess sequences necessary for interference. Further investigation is needed to resolve these results and determine the nature of this propagated RNA.

CHAPTER 8

ANALYSIS OF CLONED DI SFV IN VIVO

INTRODUCTION

This chapter describes the biological activity of cloned DI SFV preparations *in vivo*. Differences in mouse protection by the DI viruses derived from pSFVDI-6 and pSFVDI-19 is discussed in relation to the differences in their nucleotide sequences.

Although by definition all DI SFV preparations interfere *in vitro*, only some protect *in vivo*. Barrett and Dimmock (1984b) showed that while the DI SFV preparations p4 and p13a prevented disease and death from a normally lethal dose of SFV, DI SFV p5 had no such effect. These preparations had similar levels of SFV antigen and interfered with SFV *in vitro* to a similar degree. It was concluded that there was some function of DI SFV genomes relevant to their ability to protect *in vivo*. However, the DI SFV preparations used in these experiments were heterogeneous and the nucleotide sequences of the constituent genomes were not known. It was not possible therefore to determine which DI SFV genomes were responsible for the mouse-protecting activity. The previous chapter showed that homogeneous DI SFV populations could be prepared from cloned genomes and these preparations could therefore be defined on the basis of their constituent RNA. This chapter describes the effects of co-inoculation into mice of these preparations with a lethal dose of SFV, and discusses the regions of the cloned molecules that may be responsible for mouse-protection.

Mouse experiments were performed using a plaque-purified (ts^+) strain of SFV or the cloned strain of SFV that had been used in the transfection experiments. This

chapter compares the degree of mouse-protection by DI SFV against these two strains of SFV

The previous attempts to detect DI SFV genomes in the brain tissue of mice inoculated with tissue culture DI virus were unsuccessful (see Chapter 3) Although RT-PCR was used successfully to detect tissue culture DI SFV, it was not known if mouse-protecting DI SFV genomes *in vivo*, which were presumably propagated in the brain, were small enough to be amplified by RT-PCR, or if their nucleotide sequences were complementary to the primers that were used These experiments were repeated using cloned DI SFV, which was known to be amplified readily by RT-PCR This chapter describes the attempts made to detect cloned DI SFV in mouse brain following intranasal inoculation with SFV In addition, to determine if brain cells supported the propagation of these DI SFV preparations, mice were inoculated by the intracerebral route and brain RNA analysed by RT-PCR

RESULTS

I. Analysis of cloned DI SFV *in vivo*

(a) Methodology

For these experiments, CFLP, CD-1 or Balb/c mice were used to determine strain susceptibility to DI SFV and partly depending on availability. All strains were equally susceptible to infection by SFV. Cloned SFV preparations were analysed in mice by intranasal co-inoculation with a lethal dose of SFV (10 LD_{50} = 1500 p.f.u. ts^{1} strain of SFV, or 4000 p.f.u. cloned SFV). Infectivity was removed from the DI SFV preparations by u.v.-irradiation (see Methods). As negative controls, groups of mice were inoculated with u.v.-irradiated DI SFV or culture medium. As a positive control, DI SFV-p7 was used, which had been produced by 7 serial undiluted passages in BHK-21 cells and typically gave 60-80% protection against the ts^{1} strain of SFV (N.J. Dimmock, unpublished data). As described in Chapter 3, this was the DI SFV preparation from which the clones pSFVDI-6 and pSFVDI-19 were derived.

It should be noted that in preliminary experiments DI SFV preparations were only investigated for mouse-protecting activity and controls for antigen-mediated protection were not included. In addition, the concentration of DI SFV in different preparations was not standardised, so it is not possible to conclude that preparations which conferred no protection comprised non-protecting DI SFV.

(b) Experiments using cloned SFV

In preliminary experiments, SFV derived from the full-length clone pSP6-SFV4 was used (vSFV4-p2/F13). This has similar pathogenic properties to the L10 strain (Glasgow *et al.*, 1991). Table 8.1 shows a comparison of the ability of different DI SFV preparations to modulate infection of mice inoculated with a lethal dose of SFV. These results are compared with the relative concentrations and *in vitro* interference titres of the DI SFV preparations, which were discussed in Chapter 7. The estimation of relative concentrations was based on a subjective assessment of quantities of PCR products derived from tissue culture preparations of DI SFV, and assumed that different DI RNAs were amplified with equal efficiency.

After inoculation with the cloned virus preparation, the mean time of death was 4 days, which was a day less than after inoculation with the ts$^+$ strain in CFLP mice (see below). In all DI SFV groups, the mean time of death did not differ significantly from that of the virus control group.

Only 20% protection was observed after inoculation with DI SFV-p7, which contrasts with 60-80% protection observed using the ts$^+$ strain of SFV. The greatest degree of protection was observed using vDI7 /p2. As stated previously, this preparation was derived from PCR product that had been generated from DI SFV-p7 (see Chapter 7). This process resulted in the enrichment of a 1.2 kb DI SFV genome that was suspected to be the same as that constituting pSFVDI-19. However, the number of survivors after inoculation with vSFVDI-19/p3 was only 30%, despite evidence from RT-PCR analysis that the 1.2 kb DI SFV genome was more abundant in this preparation. Further, the *in vitro* interference titre of

Table 8.1 *Assessment of mouse-protecting ability of cloned DI SFV preparations using cloned SFV.* *

Group	Relative concentration †	Relative interference titre ‡	% Survival §
virus alone	-	2	0
DI SFV-p7	+	128	20
vDI7 /p2	+	4	40
vSFVDI-19 /p3	+++	64	30
vSFVDI-19 /p7	+++	32	20
vSFVDI-6 /p3	++	16	0
virus [g!!]	+++	64	0

* All DI SFV preparations were u.v.-irradiated for 80 s; this removed infectivity but not interference in cell culture. SFV derived from pSP6-SFV4 was used (Liljeström *et al.*, 1991). Male CFLP mice (5 weeks of age) were coinoculated with 10 LD_{50} SFV and DI SFV intranasally, in groups of 8 or 10. Control groups inoculated with medium, or u.v.-irradiated DI SFV alone showed no signs of clinical disease throughout the course of the experiments (10 days).

† These were determined by visual examination of PCR products derived from tissue culture DI SFV preparations on an agarose gel (see Table 7.1).

‡ The *in vitro* interference titres were determined by tissue culture assay (see Table 7.1). Numbers refer to 1/highest dilution of DI SFV preventing c.p.e. at 24 hr.

§ No mice survived after inoculation with SFV alone and it was therefore not necessary to correct these values.

vSFVDI-19 was significantly greater than that of vDI7 /p2. It is also interesting to note that vDI7 /p2 conferred better protection than the positive control (DI SFV-p7) despite having a much lower interference titre.

There was no significant difference in protecting activity between passages 3 and 7 of vSFVDI-19. Chapter 7 showed that similar PCR products were generated from different passages of vSFVDI-19 and the constituent DI SFV genome was 1.2 kb in all samples. However, different passages and preparations of vSFVDI-19 had different interference titres *in vitro* (data not shown). This variation was suspected to be a consequence of slight differences in the conditions used to passage the DI virus and may account for the marginal differences in protection.

There was no protection after inoculation with vSFVDI-6. Although the DI SFV concentration in this preparation was not standardised against the positive control, the interference titre *in vitro* was significantly greater than that of vDI7 /p2, which conferred 40% protection. Finally, the virus control used in the transfection experiments (passage 9) was included because it had a high interference titre *in vitro*, and thus was a control for putative mouse-protecting DI SFV generated *de novo* in the transfection experiments. No protection was observed after inoculation of this DI SFV preparation.

(c) Experiments using the ts^{+} strain of SFV

Table 8.2 shows two mouse-protection experiments using the ts^{+} strain of SFV. The mean time of death was 5 days for CFLP mice and 7 days for Balb/c mice. These values did not vary significantly between virus-alone and DI SFV groups.

Table 8.2 *Assessment of mouse-protecting ability of cloned DI SFV preparations using the ts⁺ strain of SFV.* *

Group	Relative concentration †	Relative interference titre ‡	% Survival (corrected) §	
			CFLP	Balb/c
virus alone	-	2	0	0
DI SFV-p7	+	128	30	75
vDI7 /p2	+	4	75	nd
vDI7 /p3	+	4	44	nd
vSFVDI-19 /p2	+++	64	nd	88
vSFVDI-19 /p3	+++	64	58	44
vSFVDI-6 /p3	++	16	0	nd

* All DI SFV preparations were u.v.-irradiated for 80 s, this removed infectivity but not interference in cell culture. Mice were coinoculated with 10 LD_{50} SFV (ts^+) and DI SFV intranasally, in groups of 7-9. Control groups inoculated with medium, or u.v.-irradiated DI SFV alone showed no signs of clinical disease throughout the course of the experiments (10 days).

† These were determined by visual examination of PCR products derived from DI SFV preparations on an agarose gel (see Table 7.1).

‡ The *in vitro* interference titres were determined by tissue culture assay (see Table 7.1). Numbers refer to 1/highest dilution of DI SFV preventing c.p.e. at 24 hr.

§ The percentage of mice surviving after inoculation with SFV alone was 13% (CFLP) or 0% (Balb/c), and was subtracted from those surviving after inoculation with SFV + DI SFV.

nd = not done

The positive control sample (DI SFV-p7) used in the experiment with CFLP mice had been re-frozen and thawed before inoculation. This process is likely to have damaged some of the DI SFV particles, and protecting activity may have been reduced compared with the sample used in the experiment with Balb/c mice for this reason.

As with the experiment described using cloned SFV, both vDI7 and vSFVDI-19 protected and vSFVDI-6 did not. However, the degree of protection was significantly higher using the ts^a strain of SFV. The experiments shown in Table 8.2 further support the observation that although vDI7 conferred a similar degree of protection as vSFVDI-19, the data from RT-PCR and interference assay suggested that this preparation contained a lower concentration of DI SFV.

Different passages of vDI7 protected to different degrees. Chapter 7 showed by RT-PCR analysis that passage 2 consisted predominantly of a 1.2 kb DI SFV genome and passage 3 was heterogeneous. This change was reflected in the reduction of mouse-protection from 75% (passage 2) to 45% (passage 3).

(d) Investigation of immune-mediated protection by cloned DI SFV

To investigate the possibility that protection was immune-mediated, mice were inoculated with vSFVDI-19 pre-treated with β-propiolactone (BPL). BPL destroys the infectivity of viruses by interacting with their nucleic acid and without significantly altering the properties of their proteins (Barrett *et al*., 1984d). For this experiment, CD-1 random-bred mice were used at 5 weeks of age in groups of 8 or 10.

Table 8.3 shows that following intranasal inoculation of 10 LD_{50} SFV + untreated vSFVDI-19 (passage 2), 75% mice survived and only 10% survived inoculation with SFV alone. There was no significant difference between the number of survivors in the virus control group and groups inoculated with 10 LD_{50} SFV + BPL-treated vSFVDI-19/p2.

(e) Investigation of post-infection immunity in mice inoculated with SFV + cloned DI SFV

Mice that were protected from a normally lethal SFV infection with DI SFV were challenged by intranasal inoculation of 100 LD_{50} SFV (1.5×10^4 pfu) to determine if they had developed a protective immune response. All mice died of clinical disease that followed its normal course. In addition, mice that had been inoculated with DI SFV and u.v.-inactivated SFV were susceptible to challenge.

(f) Propagation of DI RNA in the brains of mice co-inoculated with SFV and cloned DI SFV.

In order to determine if vSFVDI-6 and vSFVDI-19 were propagated in the brain, mice were co-inoculated with 100 LD_{50} SFV (ts*) and DI SFV, by the intranasal or intracerebral routes. After intranasal inoculation, brains were dissected from mice in the advanced stages of the disease (usually 4-5 days after infection). Following intracerebral inoculation, brains were dissected after 1 hour (d0) and then daily. RT-PCR was used to detect DI sequences in RNA extracted from brains, with primers specific for the termini of SFV (*5'SFV* + *3'SFV*). It should be noted that in

Table 8.3 shows that following intranasal inoculation of 10 LD_{50} SFV + untreated vSFVDI-19 (passage 2), 75% mice survived and only 10% survived inoculation with SFV alone. There was no significant difference between the number of survivors in the virus control group and groups inoculated with 10 LD_{50} SFV + BPL.

(e) Investigation of post-infection immunity in mice inoculated with SFV + cloned DI SFV

Mice that were protected from a normally lethal SFV infection with DI SFV were challenged by intranasal inoculation of 100 LD_{50} SFV (1.5×10^4 p.f.u.) to determine if they had developed a protective immune response. All mice died of clinical disease that followed its normal course. In addition, mice that had been inoculated with DI SFV and u.v.-inactivated SFV were susceptible to challenge.

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Table 8.3 *Investigation of immune-mediated protection by cloned DI SFV.* *

Group	% Survival
virus alone	10
non-infected	100
vSFVDI-19 alone	100
DI SFV-p7	50
vSFVDI-19 /p2	75
† BPI -treated vSFVDI-19 /p2	0

* All DI SFV preparations were u v -irradiated for 80 s, this removed infectivity but not interference in cell culture Adult CD-1 mice were co-inoculated intranasally with 10 LD_{50} SFV (ts* strain) + DI SFV in groups of 8

† vSFVDI-19 was treated with 0 01% (v/v) BPI. to inactivate the nucleic acid

these experiments the concentration of vSFVDI-6 was lower than that of vSFVDI-19 (see Chapter 7)

When mice were inoculated intranasally, neither vSFVDI-6 or vSFVDI-19 could be detected However, after intracerebral inoculation, vSFVDI-19 was detected at 2,3 and 4 days (Figure 8 1) vSFVDI-6 was not detected in brain samples taken up to 2 days after inoculation As a control, the same volume of vSFVDI-6 as used in the inoculum was added to the homogenate of one brain from an SFV-infected mouse prior to RNA extraction, and this was readily detected by RT-PCR In addition, vDI7 /p2 was not detected by RT-PCR in brains dissected up to 2 days after infection, although it protected mice to a greater degree than vSFVDI-19 (see Tables 8 1 and 8 2)

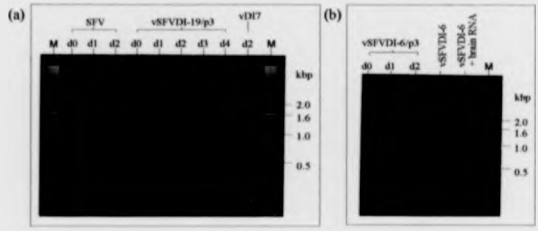

Figure 8.1 Investigation of the propagation of cloned DI SFV in mouse brain

Mice were inoculated intracerebrally with 100 LD_{50} SFV and DI SFV in a total volume of approximately 50µl. Brains were removed and RNA extracted using guanidinium isothiocyanate and analysed by RT-PCR using primers specific for the termini of SFV RNA (*5'SFV* + *3'SFV*). In **(a)**, a 1.2 kbp product was generated from samples taken 2, 3 and 4 days (d2, d3, d4) after inoculation with SFV + vSFVDI-19. DI SFV was not detected in samples taken after inoculation with SFV-alone or SFV + vDI7. In **(b)** DI SFV was not detected in samples taken after inoculation with vDI SFV-6. However, vSFVDI-6 (2.1 kb) was detected in the inoculum and in a brain homogenate to which 80µl vSFVDI-6 had been added before RNA extraction. The 0.6 kbp band in all lanes probably derived from non-specific hybridisation of the primers to SFV RNA or to mouse RNA.

DISCUSSION

The experiments described in this chapter showed that vSFVDI-19 had mouse-protecting activity However, to confirm that vSFVDI-6 was non-protecting it will be necessary to standardise the concentration of DI SFV in this preparation Evidence from dot blot analysis (see Chapter 7) suggested that the concentration of DI SFV in vSFVDI-6 was lower than that of vSFVDI-19 However, vSFVDI-6 had a higher interference titre *in vitro* than the mouse-protecting preparation vDI7 (see Table 8 2), suggesting that vSFVDI-6 was indeed non-protecting

The strain of SFV was an important determinant of protection *in vivo*, and protection by certain preparations of DI SFV was lower against SFV derived from the full-length clone than against the ts* strain (see Tables 8 1 and 8 2) Previous work showed that the protection also varied with different plaque-purified preparations of the ts* strain of SFV (N J Dimmock, unpublished data) These results suggest that highly specific interactions occur between standard and DI virus *in vivo*, which can be affected by slight differences in the nucleotide sequence of the standard genome

Different preparations and passages of vSFVDI-19 protected to different degrees It is likely that the concentration of DI SFV in these preparations was at a threshold required for mouse-protection, and that a slight reduction in this resulted in a disproportionate loss of mouse-protecting activity In a preliminary experiment, a three-fold dilution of vSFVDI-19 abrogated protection (data not shown) Previous work by Barrett and Dimmock (1984c) showed that two-fold dilution of DI SFV (p4 and p13a) resulted in a disproportionate loss of mouse-

protecting activity. They concluded that the ratio of standard to DI virus was a critical factor in protection. It will be interesting to determine if the degree of protection is increased by concentrating vSFVDI-19. However, this may result in sufficiently high quantities of antigen to elicit immune-mediated protection.

Protection was not observed after inoculation with BPL-inactivated vSFVDI-19. This shows that protection required the participation of a biologically active genome and was not immune-mediated, supporting evidence from previous work which showed that protection *in vivo* by DI SFV was independent of the amount of associated antigen (Dimmock and Kennedy, 1978; Barrett and Dimmock, 1984a, b, c). Mice protected using vSFVDI-19 were not resistant to challenge. Previous work by Barrett and Dimmock (1984b) showed that while mice co-inoculated with SFV and some preparations of DI SFV were completely immune to subsequent lethal challenge with SFV, mice inoculated with SFV and other preparations of DI SFV had no immunity whatsoever. This was despite of the fact that SFV multiplied in the CNS and systemically to the same extent in all groups of mice.

Barrett and Dimmock (1984b) also showed that after co-inoculation of DI SFV with encephalomyocarditis (EMC) virus, which is sensitive to interferon (Gresser *et al*., 1976), no modulation of EMC virus infection was observed. Protection was therefore not suspected to be mediated by non-specific immune responses. Similar experiments need to be performed with vSFVDI-19 to confirm that protection resulted from intracellular interference of DI SFV with standard virus and not from non-specific immune responses.

vDI7 /p2 protected mice to the same degree as the positive control (DI SFV-p7), and generally better than vSFVDI-19, despite evidence from an *in vitro* interference assay that the DI SFV concentration was much lower in this preparation (see Tables 8.1 and 8.2). However, passage 3 of vDI7 had reduced protecting activity and this may have been the consequence of the loss of a 1.2 kb DI SFV genome (see Chapter 3). These results support previous conclusions that mouse-protection by DI SFV is mediated by specific DI RNA sequences (Barrett and Dimmock, 1984b).

Evidence from restriction analysis of PCR products derived from vDI7 and vSFVDI-19 suggested that the 1.2 kb DI SFV genome was the same in both (see Chapter 7). It is possible that the difference in mouse-protecting activity between the preparations lies at the level of individual nucleotides. Such differences would not alter the primary structure of the genome significantly but may result in alteration of a region of secondary structure involved in the intracellular interactions with standard virus RNA *in vivo*. For example, nucleotide 22 of pSFVDI-19 is thymine and in SFV RNA it is cytosine (see Chapter 5). This substitution is within a 44 nucleotide region that forms a stem-loop structure and is conserved at the 5' termini of different alphaviruses (Ou *et al*., 1983). Furthermore, Taiang *et al* (1988) showed that by deleting 11 nucleotides in this region of a DI Sindbis clone (DI-549), this stem-loop structure was disrupted and the genome was inactivated. To determine if single nucleotide differences in pSFVDI-19 resulted in decreased mouse-protecting activity, compared with the genome within the original DI SFV preparation, it would be necessary to re-clone. However, vSFVDI-19 clearly had mouse-protecting activity and will permit future investigation into the sequence-function relationships of DI SFV.

Following intracerebral inoculation of mice with a mixture of SFV and cloned DI SFV populations, vSFVDI-19 was propagated and vSFVDI-6 was not. To confirm these conclusions it will be necessary to repeat the experiment using standardised concentrations of DI SFV and taking samples from mice 3 and 4 days after inoculation with vSFVDI-6. However, an explanation as to why vSFVDI-6 did not protect against a lethal dose of SFV and vSFVDI-19 did could be that the former is not propagated *in vivo*. The synthesis of alphavirus genomic and sub-genomic RNAs is believed to require specific *cis*-acting sequences and a combination of virus-specific proteins and host cell components that act in *trans* (Grakoui *et al.*, 1989). It is possible, therefore, that vSFVDI-6 is propagated *in vitro* but not *in vivo* because the replicase complex formed in brain cells differs from the complex formed in BHK-21 cells.

Chapter 5 showed that pSFVDI-6 possesses all the nucleotide sequences present in pSFVDI-19. It is likely, therefore, that the reason vSFVDI-6 had no biological activity *in vivo* compared with vSFVDI-19 was because its additional sequences (which comprised 0.9 kb) abrogated protection. It is possible that these sequences disrupted secondary structure that is involved in intracellular interactions with the standard genome or replicase complex *in vivo*. Such interactions may be dependent on the size of the DI SFV genome or the length of sequences separating protein-binding domains.

CHAPTER 9

GENERAL DISCUSSION

This thesis describes the development of a system that was used to generate genetically homogeneous preparations of DI SFV and identify nucleotide sequences important for the biological activity of DI SFV genomes both *in vitro* and *in vivo*. As specific experimental data have been discussed already, this final section will provide an overview of the results obtained and speculate on the implications of this work for future studies.

The RT-PCR technique provided an simple and effective means for isolating DI SFV genomes from heterogeneous tissue culture preparations. This technique was also useful for studying the progression of DI SFV genome populations on serial passage. For example, Figure 3.6 showed that a 1.2 kb DI genome was replaced by a 2.1 kb genome after a single passage with the concomitant loss of mouse-protecting activity. This confirms previous observations that DI SFV genome populations can increase in size on passage (Kääriäinen *et al*., 1981) and suggests that smaller DI genomes are not necessarily more competitive. It is possible that DI genomes that interfere strongly with standard virus propagation are less successful, since a DI genome that interferes strongly with helper virus will do so at its own expense. However, this situation is unlikely to arise during passaging at a high-multiplicity of infection. Clearly the replicative advantage of DI SFV genomes lies with a function of the nucleotide sequence but may also be dependent in part on other factors, such as the host cell components involved in viral RNA synthesis.

Two DI SFV genomes were cloned and sequenced (pSFVDI-6 and pSFVDI-19). Both were essentially unaltered subsets of the virion RNA, thus differing from previously sequenced DI SFV genomes which comprised repeated and rearranged regions of the standard genome (Lehtovaara *et al*., 1981, 1982). These findings

suggest that different mechanisms are involved in the generation and amplification of DI SFV genomes However, pSFVDI-6, pSFVDI-19 and the clones described by Lehtovaara and associates were all derived from similar regions of SFV RNA suggesting a common sequence requirement for the production of DI SFV

It is not possible from the sequence data alone to deduce the mechanisms involved in the generation of the RNA from which pSFVDI-6 and pSFVDI-19 were derived Both these molecules could have been generated *de novo* from the standard genome or one was generated from the other pSFVDI-6 contains all the sequences of pSFVDI-19 and the latter could therefore have been created through deletion of the former However, evidence from RT-PCR of the tissue culture fluid from which the two clones were derived suggested that pSFVDI-19 RNA predominated in passage 7, while pSFVDI-6 RNA predominated in passage 8, implying that pSFVDI-19 RNA was not generated through sequential deletion events Alternatively the RNA from which pSFVDI-19 was derived may have been of a structure that favoured recombinational events with the standard RNA, thus generating the larger RNA from which pSFVDI-6 was derived If this was the case, it suggests that the interfering activity of pSFVDI-19 RNA may result in part from some type of recombinational ability that would presumably reduce the 42S RNA population in the infected cell However, the stability on serial passage of the cloned preparation of DI virus derived from pSFVDI-19 (discussed below) suggests that this is unlikely

It is probable that the secondary structure of a DI genome is important to its biological functions As stated previously, SFV RNA can circularise and form panhandle structures under the conditions used for electron microscopy (Hsu *et al*.,

1974, Frey *et al*., 1979) Both pSFVDI-6 and pSFVDI-19 have retained the regions from within the termini of SFV RNA proposed by Ou *et al* (1983) to be involved in the base-pairing of these panhandles, and may therefore be capable of circularising or concatamerising Indeed, the northern blot analysis of RNA derived from vSFVDI-19 suggested that at least under certain conditions the molecule can form multimers (see Figure 7 6) Although it is difficult to envisage what role genomic circularisation may have in the infected cell, it is possible that complementary regions within the genome may permit intergenomic base-pairing Such an event may cause errors to be made by the viral replicase and hence explain how novel DI genomes can be generated from existing ones Although this is speculation, a number of DI influenza virus genomes have been shown to contain regions derived from different segments of the standard genome This suggests that the generation of DI genomes can, at least in the case of influenza virus, arise through intermolecular recombinational events (reviewed in Nayak *et al*., 1989)

Clearly there are many regions of the cloned DI SFV genomes that could be involved in secondary structure formation Some of these regions may be essential to the propagation of the DI genome as virions or to the replicative advantage of a particular DI genome, while others may only be incidental and have no biological function Interference by DI SFV genomes may be mediated through secondary structure interactions with the viral replicase complex or through other mechanisms Both pSFVDI-6 and pSFVDI-19 encoded polypeptides in an artificial translation system (see Figure 6 5) Whether such polypeptides are produced in the infected cell or contribute to interfering activity remains to be determined

One of the more important achievements described in this thesis was the development of a system that permitted the production of genetically homogeneous preparations of DI virus. Transfection of RNA transcripts from cloned DI SFV genomes into SFV-infected BHK-21 cells by electroporation generated detectable quantities of DI virus after a single passage. It is likely that the high efficiency of the electroporation procedure prevented the establishment of DI genomes generated *de novo* and may partly explain the stability on passage of the cloned DI virus preparations. While the transfection procedure was used primarily to generate cloned DI virus populations, it was also used in a preliminary investigation into the functional regions of the cloned DI genomes. A region within the nsP2 gene of SFV RNA (nucleotides 2637 to 3024) was tentatively defined as containing a packaging signal. This region is conserved in pSFVDI-6, pSFVDI-19 and the DI genomes described by Lehtovaara *et al* (1982), and clones which lacked this region (pSFVDI-M2 and pSFVDI-SB1) were not propagated as virions. Further experiments are needed to resolve the precise function of this region of the SFV genome, but these experiments underline the usefulness of DI genomes for the identification of viral regulatory elements.

With the establishment of genetically homogeneous preparations of DI SFV it was possible to investigate the relationship between biological activity and genome constitution. Evidence from dot blot analysis showed that pSFVDI-6 was not propagated in tissue culture as efficiently as pSFVDI-19. For future comparative studies it will therefore be necessary to standardise the concentration of DI SFV in these two preparations.

After intranasal coinoculation of the cloned preparations of DI SFV and 10 LD_{50} SFV into adult mice, vSFVDI-19 typically conferred about 75% protection and vSFVDI-6 was non-protecting. Modulation of infection was not observed after intranasal coinoculation of BPL-inactivated vSFVDI-19 with a lethal dose of SFV, showing that protection was independent of the amount of antigen. This supports previous evidence that protection by DI SFV is a function conferred by the genome and is not mediated through interactions with the host immune system (Dimmock and Kennedy, 1978; Barrett and Dimmock, 1984a, b, c; Barrett *et al*, 1984c). Although the DI SFV concentration in these preparations was not standardised, the tissue culture preparation vDI7-p2 typically conferred about 80% protection, despite having a lower interference titre than vSFVDI-6. This provided further evidence that vSFVDI-6 was non-protecting, and confirmed previous work which showed that only some DI SFV preparations have mouse-protecting activity (Barrett and Dimmock, 1984a, b).

The propagation of the two cloned DI SFV preparations in mice was investigated. Evidence from PCR analysis of RNA extracted from the brains of DI SFV-treated mice suggested that only vSFVDI-19 was propagated. It will be necessary to standardise the concentration of DI SFV in the two preparations to confirm this result, but it does provide a simple explanation for why some DI SFV preparations are mouse-protecting and others are not. This experiment also suggests that interference is dependent in part on host cell factors. It is possible that the replicase complex formed in brain cells differs from that formed in tissue culture in such a way as to prevent certain DI genomes from being propagated. It is also possible that only certain types of cell in the brain are involved with the propagation of DI SFV. It will be interesting to determine if interference only occurs in specific areas

of the brain or olfactory lobes after inoculation by the intranasal route, but to do this will require the development of a technique that is sufficiently sensitive and specific to detect DI SFV *in vivo*. Although RT-PCR failed to detect DI SFV in mouse brain after intranasal administration, this may have been because the protocol used was not sufficiently sensitive. It may be possible to increase sensitivity and specificity by using a nested PCR technique, which is feasible now that a mouse-protecting DI SFV clone of known sequence has been constructed.

Finally it is pertinent to ask which regions of a DI SFV genome are important for mouse protection. Clearly certain regulatory sequences have to be retained by the DI genome for viability, but whether other sequences are required for a specific interaction with virus replication *in vivo* is not known. pSFVDI-6 contains all the sequences of pSFVDI-19 and is apparently non-protecting, suggesting that mouse protection is not mediated through interactions with the primary structure of the DI genome. However, it is possible that the additional sequences of pSFVDI-6 are involved in secondary structure formation that may mask regions required for interference *in vivo*. Alternatively it may be the molecule as a whole that is important for mouse protection. Hence factors such as size, coding potential, distribution of regulatory elements along the molecule or overall secondary structure may be important to interference *in vivo*. Clearly further work is needed to determine which elements of pSFVDI-19 are required for modulating infection *in vivo*, and this aim could readily be achieved using the methodology described in this thesis.

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APPENDIX

NOMENCLATURE USED FOR MOLECULAR CLONES, DI VIRUS AND VIRUS PREPARATIONS

LIST OF MOLECULAR CLONES

Clone	*Vector*	*Size of insert (kbp)*	*Derivation*
pSFVDI-6	pUC13	2.1	RT-PCR amplification from DI SFV-p8
pSFVDI-7	pBluescribe KS$^+$	2.1	As pSFVDI-6
pSFVDI-19	pUC13	1.2	RT-PCR amplification from DI SFV-p7
pSP6-SFV4	pTZ18R	11.4	SFV cDNA (Liljeström *et al.*, 1991)
pSFVDI-1A2	pBluescribe KS$^+$	1.2	deletion of pSFVDI-6 from AccI 534 to AccI 1453
pSFVDI-M2	pUC13	1.7	deletion of pSFVDI-6 from MluI 1225 to MluI 1690
pSFVDI-SB1	pTZ18R	2.1	deletion of pSP6-SFV4 from SalI 1658 to BstEII 10920

LIST OF VIRUS AND DI VIRUS PREPARATIONS [a]

Name	*Derivation*
DI SFV-p7	7 serial undiluted passages of ts$^+$ SFV in BHK-21 cells
DI SFV-p8	1 undiluted passage of DI SFV-p7 in CEF cells
vDI7-p2	RT-PCR amplification from DI SFV-p7 using the primers *5'SFV-T3* + *5'SFV-Nco*, followed by transcription, transfection and 2 serial undiluted passages in BHK-21 cells
vSFV4-p2/F13	transfection of RNA transcribed from pSP6-SFV4 followed by one serial passage in BHK-21 cells at an m.o.i. of 0.01

[a] tissue culture preparations derived from transfection of RNA transcribed from molecular clones are prefixed 'v'